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PROCEEDINGS
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SIGN AND SYMBOL IN BEE COMMUNICATIONS

By A. L. Kroeber

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Read before the Academy April 28, 1952

Signs or signals are distinguished from symbols as means of communication, both in their mechanism and in their function.

They differ in their mechanism in that signs are primarily genetic in origin, and therefore basically congenital in the species, though the ability to produce them or react to them may be dependent on maturation, and there may also exist a supplementary degree of learning by individual experience. Communicative symbols, on the contrary, are wholly learned, subject only to minor extensions by such newly originated symbols as may be accepted by other individuals.

In function, signs that serve to communicate are action responses to sensory or visceral stimuli and are probably always accompanied by emotional affects. Directly, they convey information to recipient individuals only as to the condition of the sign-producing individual. They alert one organism as to the condition of another, which is often useful. True symbols, however, can convey information on other matters than the condition of the communicating organism. Such external information may fairly be called objective, as compared with the essentially subjective nature of what is communicated by non-symbolic signs.

Once having crossed the threshold into communicability by words of objective reference, even a still rudimentary system of symbolism possesses the possibility of indefinite extension on its level. Not only can it convey specific information, but it can proceed to conceptualize, to generalize and to abstract. Therewith institutions, judgments, and culture are made possible. The beginnings of symbolic communication accordingly mark a critical point in the history of our planet—the emergence of basic human faculty.

Von Frisch's classic studies of the "language" of honey bees[1] deal with communications among the members of a species which—like all other subhuman species—has been believed to be wholly non-symbolizing.

However, he has shown conclusively that these intercommunications of bees successfully convey information on certain matters usually considered abstractions—especially distances and directions—which have hitherto been believed communicable only by true language, viz., symbolic speech or its visual derivatives. On their face, the von Frisch observations and experiments thus seem to establish a phenomenon of revolutionary novelty.

I have discussed the facts with several linguists. Few of them would accept the honey bee accomplishment as being symbolic and as therefore presenting at least the rudiments of a system truly constituting a "language," —however different from our own it might be in its specific means or scope.[2] The linguists seemed to take the attitude that the bees' informations must be mere signs or signals; they scarcely tried to analyze away the symbolic nature of these informations. I wish therefore to investigate the more precise nature and qualities of some of the more startling of the bee intercommunications.

When a worker bee discovers a notable supply of nectar, according to von Frisch, she executes with some excitement, on her return to the hive, a dance which the bees there follow with interest, and then proceed to visit the source. If the new source of supply lies within 50–75 meters, the dance is a circle whose direction is reversed after each circuit. The informed bees have therefore only to search in the vicinity of the hive—being guided to the correct species of flower by its scent that they have perceived adhering to the body of the scout. The richer the supply, the more vigorously the scout dances—as if abundance correspondingly stimulated her. So far, we are not beyond the realm of possible stimulus cues or signs.

If the new source of food is farther from the hive than 75–100 meters, the returned scout performs a different dance which follows a figure-eight course while the abdomen is being wagged or laterally vibrated. The dancer's speed is a function of the nearness of the supply. In a quarter minute she executes 9 to 10 full figures if she has flown in from only 100 meters; 7 if she has traveled 200 meters; $4^1/_2$, for 1000; 2 for 6000. Within about 800 meters the curve has fallen halfway from the fastest to the slowest dance tempo.

This performance might be more than a sign cue, but need not be construed so. There is no necessity for assuming intent to symbolize distance by speed of motion. Theoretically, increased fatigue might account for the decreased dance tempo. Only one subsidiary point is not clear. According to von Frisch, a head wind on the way to the food slows the tempo much as a proportionate increase of distance slows it. We should rather expect a head wind on the *return* journey to slow the dance, if fatigue is at work. After a contrary wind on the way out, the worker would recuperate while rummaging for a while on and in the flowers; after which

she would return to the hive under a favoring stern wind with even less fatigue than the distance called for on a calm day.

With increasing distance, the exact direction of the discovered supply is increasingly important to the worker's fellow-citizenesses. Heavy effort and many failures would result from their trying to find a spot 1 or 2 kilometers distant not further indicated. In meeting this directional need, the straight part of the figure-eight dance becomes crucial—the choreographic line by which the two circles are united. If the scout has a horizontal board on which to alight before an audience in front of her hive door, she may dance there; or again, inside in the unusual event of a honeycomb lying level. In either event the straight part of her horizontal dance points directly in the direction of the food source.

Normally, however, according to von Frisch, the comb surface for dancing is vertical. In that case, her procedure seems definitely symbolic. When she comes home from a food supply lying straight in the direction of the sun, the indicative part of her dance is upward, straight against gravity. In proportion as the food is situated to one side of the sun's direction, she shifts the axis of her dance from the vertical to that side. If the food direction is 60° left of the sun's direction, the axis is turned about 60° left from the vertical. For 120° right, the axis turns 120° right. This holds all the way around: if the food lies directly opposite the sun, the dancer's axis is straight downward.

This is a really remarkable phenomenon. An upward course indicates—seemingly symbolizes—the direction of the sun, wherever the sun may be at that hour. This is as arbitrary a relation as that between the sound of the word *horse* and the animal, or the sound of *hold* and the act of the verb. Moreover, a departure from verticality indicates not only the side but the *degree* of departure of direction. These dance representations certainly express relations; and relations to us are abstractions, and our representations of abstractions are by symbols.

Moreover, the indications conveyed are effective. They lead the informed bees correctly, or nearly so. At 200 meters, von Frisch set out six properly scented but empty stations, 15° apart, three on each side of the actual food source. Half the bees that responded to the dancer flew straight to the correct spot; three fourths of the others lit on one of the two nearest stations, 15° off; only one-eighth of the total number miscarried by 30 or 45°. This is quite possibly greater accuracy than could be obtained by purely verbal instructions given to human beings without reference to landmarks—especially if reference to visual symbols like hands of the clock were also precluded.

If we knew that bees individually acquired this system of direction indications from one another by learning, I believe that even the most isolationist linguists would accept the communication as constituting part

of a symbolic or true language. We have no evidence whatever that this is so. Von Frisch's skilful experiments have not been directed toward this problem of how the system is acquired by individual bees. Everything else we know about the social insects points to the strong preponderance of genetic factors over individual experience and learning in their ontogeny. I presume that is why the negative reactions of most of my linguistic consultants have been so clear-cut. Insects are not known to transmit knowledge by learning; hence these particular communications cannot be symbolic; the necessary mechanism is lacking—so goes the argument.

However, this reasoning of course does not really prove that a symbolizing mechanism is actually lacking. Bees may be communicating by symbols learned from one another, or again, conceivably, transmitted in them genetically.

This last possibility constitutes our real problem. But until new observations or experiments become available, we can only try to analyze the situation further. First, the hypothetical bee language operates through the sense of sight, supplemented by smell; our speech of course operates—originally at least—by sound. Not only the instruments but also the content of the two systems of communication might therefore be expected to differ considerably.

Second, the bee communicates by movements of her whole body; we, by combinations of the several organs between lungs and lips.

Third, the as yet discovered bee "vocabulary" is not only quite small, but it is limited in scope. Its known reference is to food—to its abundance, distance, and direction. These are all traits which have quantitative aspects—to us, at least. Qualitative or substantive features—such as the species of flower involved—are not communicated by either sign or symbol but by odor actually brought into the hive by body adherence.

That the ability to communicate the specific information about food would possess a high selective survival value if the communicability became genetically established is not an argument which it is fair to press at present, because the problem after all is whether it did or did not become genetically established. However the fact that in general bee faculties are overwhelmingly genetic in nature sets a presumption against acceptance of a non-genetic mechanism without the closest scrutiny and test.

The general structural pattern of the social insects has changed so little, on the whole, in some tens of millions of years that if the bee communications about nectar are symbolic and non-genetic in kind, they would certainly have had abundant time to develop into a system of some degree of richness and variety. Actually their range remains narrow and meager. By contrast the symbolic languages of men are always changing in their phonic constituents, patterns of structure, and referends or meanings; and the poorest of them, though much younger, are infinitely richer and more diverse than the few scraps known from the bees.

This impermanence of human forms of speech is one facet of an inherent plasticity of which another aspect is the admitted ability of human languages to adapt rapidly to new cultural demands. Not only do languages change in adaptation to utilitarian demands, they seem to change in response to very minor internal stimuli so as to seem to alter almost "spontaneously." In these fluctuating alterations, meaning is subject to variation at least as actively as speech form. To put it more broadly, symbolic content is as subject to change as symbolic form, in human language. Once given a true symbol system of communications, one symbol can and dose breed others on very slight provocation—on stimuli much smaller than those possessing survival value.

These several differences do not eliminate the possibility that social insects communicate with symbol systems; but they do reduce its probability. If so, we may conclude, provisionally, that the seeming abstractions of fact which the bees transmit to one another may be not really such, but only seem so to us. They perhaps are pseudo-abstractions, something like the setter pointing at the bird. Unquestionably they are more complex than the dog's act, since they are indirect: the angle of departure from verticality only *corresponds* to the angle of departure from the direction of the sun. It is this indirection or arbitrariness of expression that gives to the phenomenon the appearance of abstraction in our minds.

While this interpretation seems at present the most likely, I do not consider it as definitely established. More evidence, especially experiment, may confirm or may reverse it. Von Frisch has opened a brilliant and promising path with bees. By comparison, ants eat more varied foods, wage wars, plant so-called gardens, raise or at any rate cherish live stock, are divided into castes; and termites at least live in fully bisexual societies. Whether the communication techniques of these other orders and suborders of insects are genuinely or pseudo-symbolic, they will no doubt be of extreme interest and significance when more fully and exactly known.

[1] Von Frisch, K., *Bees: Their Vision, Chemical Senses, and Language*, Ithaca, 1950.

[2] However, J. Lotz has compared bee communication and human speech in "Speech and Language," *J. Acoustical Soc. Am.*, **22**, 712–717 (1950), and again in a review of von Frisch in *Word*, **7**, 66–67 (1951).

VISCOSITIES OF SOLUTIONS OF BI-BOLAFORM ELECTROLYTES*

BY PAUL GOLDBERG AND RAYMOND M. FUOSS

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Read before the Academy April 28, 1952

Bolaform electrolytes[1] are defined as electrolytes containing ions in which at least two charged sites are joined by a chain of neutral atoms. The adipate ion, $^{-}O_2C(CH_2)_4CO_2^{-}$ is a typical bolaform anion, and bis-quaternary ions such as $Me_3N^{+}(CH_2)_nN^{+}Me_3$ are cations of this structure. Conductance measurements[1] have shown that, in solvents of intermediate dielectric constant such as methanol and ethanol, there is an appreciable association[2] of anion to one end of a bis-quaternary cation, due to the fact that the Coulomb potential is large compared to kT. If bi-bolaform electrolytes (i.e., those with both anion and cation of the bolaform type) are considered, one might therefore expect electrostatic association to lead to two possible structures: (1) a compact cyclic structure which may be symbolized as

$$\begin{matrix}(+ \ldots . +) \\ (- \ldots -)\end{matrix} \qquad (1)$$

or (2) an extended chain configuration

$$(+ \ldots . +)\,(- \ldots -)\,(+ \ldots . +)\,(- \ldots -). \qquad (2)$$

It would be difficult to differentiate between (1) and (2) by conductance methods, but (2) should contribute much more to viscosity than (1). We present herewith viscosity data on two bi-bolaform electrolytes in water, methanol and ethanol; several simpler electrolytes were measured as comparison substances.

Experimental.—Parent substances were tetrabutylammonium bromide (a 1-1 salt), 1,3-di-(trimethylammonium)-propane dibromide (a 2-1 salt, coded as 2_3-1, to indicate three atoms joining the charges of the cation) and 1,9-di-(trimethylammonium) nonane dibromide (coded as 2_9-1). These bromides were converted to the corresponding quaternary hydroxides in methanol by treatment with excess silver oxide; the filtered solutions were then neutralized with an exact equivalent of adipic acid in methanol, and then solids were recovered by vacuum evaporation. The products are, respectively, di-(tetrabutyl-ammonium) adipate (coded as 1-2_6); 1,3-di-(trimethylammonium) propane adipate (coded as 2_3-2_6) and 1,9-di-(trimethyl-ammonium)-nonane adipate (coded as 2_9-2_6). Solutions were made up by weight in the various solvents, and viscosities were measured at a series of concentrations at 25.00° in a viscometer[3] with water flow time of 250 seconds at minimum filling. The data are summarized in table 1, where η_{sp} is the difference between solution and solvent viscosities divided by solvent viscosity and C' is weight per cent of solute. For scale conversion, we note that $C' = \gamma M/10\rho$, where γ is moles per liter, M is molecular weight of solute and ρ is the density of the solution.

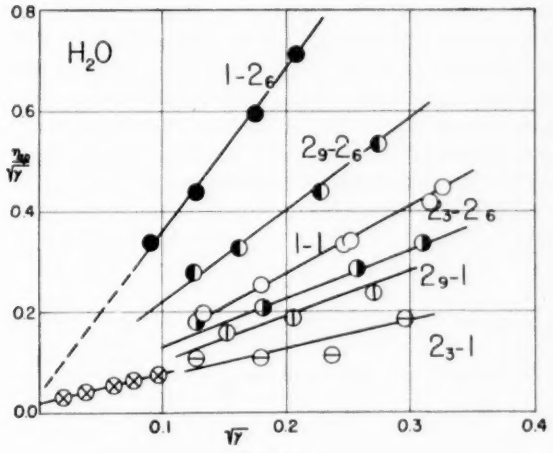

FIGURE 1.
Viscosity of salts in water.

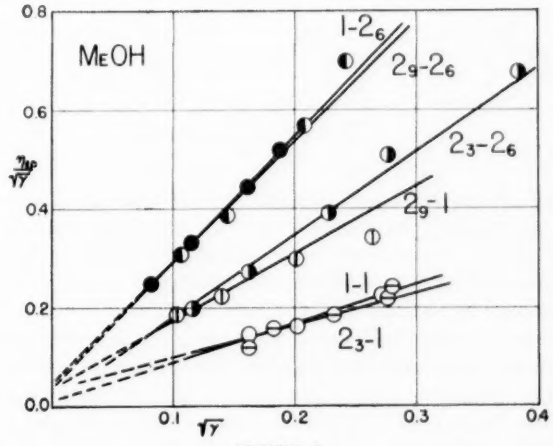

FIGURE 2.
Viscosity of salts in absolute methanol.

If reduced viscosity (η_{sp}/C') is plotted against C', all the systems give curves which become concave-up at low concentrations; the curvature indicates the presence of a long-range interaction term[4] in the viscosity. This term is proportional to the square root of ionic strength, and since it is small compared to the total viscosity increment beyond 0.01 molar for our solutions, we can estimate it sufficiently well for our present purposes, despite the fact that several of the ionic mobilities have not been determined. For a wide variety[5] of ordinary electrolytes in water, it has been found empirically that

$$\eta_{sp} = A\sqrt{\gamma} + B\gamma \qquad (3)$$

where A agrees closely with the theoretical value given by Falkenhagen.

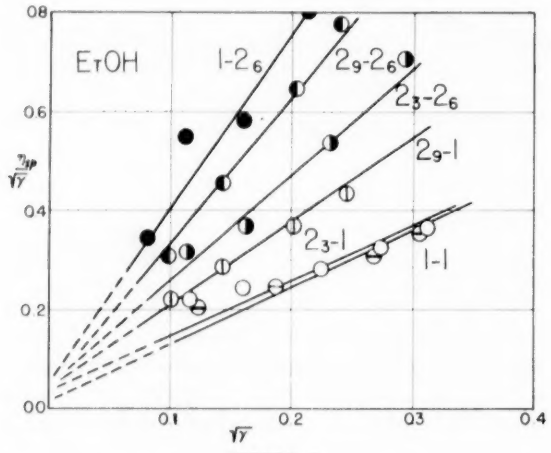

FIGURE 3.

Viscosity of salts in absolute ethanol.

The values of B are small for 1-1 salts, and for typical 2-2 salts such as $CuSO_4$ are around 0.5. In figures 1, 2 and 3 are shown plots of $\eta_{sp}/\sqrt{\gamma}$ against $\sqrt{\gamma}$ for the data of table 1. It will be noted that the plots all approximate linearity and approach as limits at $\gamma = 0$ the estimated A-values. For comparison, the data[6] for copper sulfate are included in figure 1 as the crossed circles. It is immediately obvious that the B-values for most of our salts are considerably greater than those of simple electrolytes, where the ions consist of only a few atoms. Consequently we conclude that, while the B term for ordinary electrolytes may well represent higher terms which were neglected in the mathematical theory, the empirical B's for our salts must contain something additional.

TABLE 1

VISCOSITIES OF ELECTROLYTES

WATER		METHANOL		ETHANOL	
C'	η_{sp}	C'	η_{sp}	C'	η_{sp}
		Bu_4NBr, (1-1)			
3.433	0.145_0	2.987	0.060_0	3.970	0.113_6
3.239	0.131_6	1.655	0.032_4	3.047	0.089_8
2.053	0.086_0	1.051	0.023_8	2.053	0.063_2
1.969	0.081_1	...	...	1.042	0.038_4
1.036	0.045_2	...	...	0.552	0.025_6
0.563	0.026_2	...	...	...	...
		$Br[Me_3N(CH_2)_2NMe_3]Br$, (2_2-1)			
2.728	0.053_7	3.151	0.067_8	3.811	0.109_0
1.782	0.026_8	3.076	0.060_5	2.910	0.083_4
1.029	0.019_0	2.180	0.043_1	1.429	0.045_8
0.514	0.013_4	1.348	0.028_7	0.620	0.024_7
...	...	1.063	0.019_8	...	...
		$Br[Me_3N(CH_2)_9NMe_3]Br$, (2_9-1)			
2.960	0.063_8	3.550	0.090_5	3.065	0.106_6
1.703	0.038_0	2.070	0.060_0	2.078	0.074_6
0.927	0.023_7	1.002	0.031_2	1.049	0.041_3
...	...	0.542	0.019_6	0.518	0.022_1
		$NBu_4[OOC(CH_2)_4COO]NBu_4$, (1-$2_6$)			
2.730	0.147_8	2.840	0.098_1	3.605	0.171_3
1.941	0.103_6	2.065	0.071_7	2.061	0.093_8
1.023	0.055_8	1.062	0.038_5	1.013	0.061_6
0.501	0.030_4	0.526	0.020_0	0.527	0.028_1
		$[Me_3N(CH_2)_3NMe_3][OOC(CH_2)_4COO]$, ($2_3$-$2_6$)			
2.913	0.103_5	5.913	0.302_8	3.310	0.207_1
1.982	0.073_0	2.943	0.140_8	2.055	0.123_7
0.993	0.036_6	1.996	0.089_0	1.016	0.060_4
0.496	0.022_8	1.014	0.044_4	0.503	0.036_7
...	...	0.512	0.023_1	...	...
		$[Me_3N(CH_2)_9NMe_3][OOC(CH_2)_4COO]$, ($2_9$-$2_6$)			
2.930	0.145_7	2.861	0.161_8	2.840	0.186_7
2.022	0.099_8	2.132	0.118_2	2.036	0.132_3
1.027	0.053_0	1.026	0.055_6	1.013	0.065_2
0.519	0.031_8	0.565	0.033_1	0.487	0.030_8

We next computed the quantity

$$z = (\eta_{sp} - A\sqrt{\gamma})/C \qquad (4)$$

which is $1000/M$ times the slopes B of the figures. Here C is concentration expressed in grams of solute per cc. of solution. The results of the calculation are summarized in matrix form in table 2, where, for example, z for di-tetrabutylammonium adipate in methanol is 4.0.

For rigid spherical particles in a continuum, Einstein[7] showed that

$$\eta_{sp} = 2.5vC \qquad (5)$$

where v is the specific volume of the solute. No data are available for the densities of the solutions, but the specific volumes of the solutes probably do not differ markedly from unity. If we assume that $A\sqrt{\gamma}$ is a reasonable approximation for the electrostatic term in the viscosity (and recall that it is in any case small compared to the total viscosity increment here), then

TABLE 2

HYDRODYNAMIC COEFFICIENTS z OF SALTS

Bu_4N	C_4	C_6
	Water	
Br 4.2	1.7	2.3
Ad 5.3	3.3	4.7
	Methanol	
Br 2.3	2.0	3.2
Ad 4.0	5.0	6.3
	Ethanol	
Br 3.7	3.4	4.2
Ad 5.7	6.7	7.3

we may identify z with the bulk hydrodynamic term in the viscosity, i.e., that contribution arising from the mere presence of foreign particles in the solvent. We note first that most of the systems have z values which are significantly greater than 2.5, and must (as expected) discard the simple spherical model, especially for the higher salts. A chain polymer of molecular weight 10,000 would have z approximately equal to 10 (intrinsic viscosity 0.10 in conventional polymer concentration units), so we cannot immediately rule out the possibility of chain formation by electrostatic association in the case of the bi-bolaform salts.

If the solute particle is not spherical, then

$$\eta_{sp} = \omega vC \qquad (6)$$

where ω is a coefficient which approaches 2.5 for spheres, and increases roughly as the square of the axial ratio as the sphere is distorted into an ellipsoid[8]; for an axial ratio of 5, ω is 5.8. Values in this range are found

in table 2 for *both* the 1-2 and 2-2 salts in methanol and ethanol; since the former are not likely to associate to chains, we conclude that their enhanced viscosity, and consequently that of the 2-2 salts, is due to geometrical asymmetry. Models of the ions involved are ellipsoidal structures with axial ratios in the proper range to give z-values like those found, especially in the case of the adipates where a 2-2 association would stiffen the ion pair. The 1-2 and 2-1 salts would show some flexibility, due to freedom of rotation in the $(-)\ (+\ \ldots\ +)$ or $(+)\ (-\ \ldots\ -)$ clusters, and hence would have eccentricities nearer unity, i.e., z-values nearer to 2.5.

One further argument supports the suggestion of low order association rather than chains: if we exclude the data for water, where association of any sort is improbable, a striking pattern of additivity is observed in table 2. For example, the difference between bromide and adipate is 3.0 and 3.1 for the C_3 and C_9 salts in methanol, and 3.3 and 3.1 in ethanol. Other similar matching differences are also present, which suggests that we may write

$$\eta_{sp} = \omega_1 v_1 c_1 + \omega_2 v_2 c_2. \qquad (7)$$

In other words, despite the association which is shown to take place on the basis of conductance measurements, the ions appear to contribute *independently* to the viscosity increment. Further work on this problem is in progress.

* Office of Naval Research, Project NR 054-002, Paper No. 33.

† Contribution No. 1107 from the Sterling Chemistry Laboratory.

[1] Fuoss, R. M., and Edelson, D., *J. Am. Chem. Soc.*, **73,** 269 (1951). Fuoss, R. M., and Chu, V. F. H., *Ibid.*, **73,** 949 (1951).

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[3] FitzGerald, E. B., and Fuoss, R. M., *Ind. Eng. Chem.*, **42,** 1603 (1950).

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[5] Harned, H. S., and Owen, B. B., *The Physical Chemistry of Electrolytic Solutions*, Reinhold, New York, 1943; Table (6-9-1), p. 177.

[6] Asmus, E., *Ann. Physik.*, **35,** 1 (1939).

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[8] Simha, R., *J. Phys. Chem.*, **44,** 25 (1940).

THEORY OF THE CHEMICAL REACTION RATE CONSTANT*

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Communicated by Henry Eyring, July 2, 1952

The absolute reaction rate theory as originally proposed by Eyring[1] for the theoretical calculation of chemical reaction rates from potential energy surfaces using statistical mechanics applies to reactions occurring at constant temperature and produces a reaction rate constant averaged over a thermal distribution of energies for the reactants. The limitation to thermal distribution is, of course, not essential. Recently it has become of great interest to consider the theory of reactions in which the energy distribution of the reactants varies radically from thermal. Various "hot atom" and "hot radical" reactions[2] of photochemistry,[3, 4] nuclear and radiation chemistry,[5] and such processes as the decomposition of ions in a mass spectrometer[6] furnish examples in which a more flexible form of the theory is needed.

In a natural extension of the absolute reaction rate theory one can calculate the decomposition rate of an isolated molecule which has fixed energy E by calculating the rate at which systems of a microcanonical ensemble pass through potential energy saddle points in configuration space. The energy of the ensemble is taken between E and $E + \delta E$ and initially the systems are randomly distributed in the region of phase space corresponding to the undissociated molecule. The resulting reaction rate constant[6] $k'(E)$ has the advantage that it can be averaged over any desired energy distribution. Application of this method has been discussed.[6, 7]

The derivation of the rate constant $k'(E)$ has been given[6] and will not be repeated here. The rate constant for a molecule which has one dissociation process is:

$$k'(E) = \frac{1}{h}\int_0^{E-E_a} \frac{\rho^{\ddagger}(E - E_a - \epsilon)}{\rho(E)}\, d\epsilon = \frac{W^{\ddagger}(E - E_a)}{h\rho(E)} \qquad (1)$$

where $\rho(E)$ is the number of states per unit energy interval at energy E, or is the density function[8] for the initial molecule, $\rho^{\ddagger}(E - E_a - \epsilon)$ is the density function for the activated complex, E_a is the activation energy, ϵ is the kinetic energy in the decomposition degree of freedom and finally

$$W^{\ddagger}(E - E_a) = \int_0^{E-E_a} \rho^{\ddagger}(E - E_a - \epsilon)\, d\epsilon \qquad (2)$$

is the total number of states of the activated complex for the system for all energies up to $E - E_a$.

In principle the value of $k'(E)$ can be calculated for any molecule if its potential function is known. In practice it is essentially impossible to ob-

tain an explicit expression for $k'(E)$ unless the molecule can be approximated as a number of weakly interacting independent systems which have density functions varying as a power of the excitation energy.[6] One can always make such an approximation for a molecule by taking it to be a collection of vibrators and rotators.

In order for $k'(E)$ to give the actual rate constant even approximately there must be a transfer of energy among all the various degrees of freedom which is rapid compared with the calculated decomposition rate.[6, 9] In general one expects that as excitation energy is increased from threshold values for decomposition, quantum restrictions on energy transfer between vibrational degrees of freedom should become less important and $k'(E)$ should become more valid. At extremely high energies, on the other hand, there is not sufficient time for energy transfer and the applicability of this treatment is doubtful. Formula 1 in any case is of interest as a limiting form of the rate constant, and it should usually have a range of usefulness for most unimolecular decompositions.

Throughout this discussion it is assumed that there is no reflection of reacting systems,[1] i.e., $\kappa = 1$.

1. The Relation of $k'(E)$ to $k'(T)$.—It is of some interest to see that $k'(E)$ averaged over the thermal energy distribution yields the ordinary reaction rate expression of the absolute reaction rate theory. The rate constant for temperature T is obtained by the average:

$$k'(T) = \frac{\int_0^\infty k'(E)\rho(E) \exp(-E/kT)\, dE}{\int_0^\infty \rho(E) \exp(-E/kT)\, dE} \tag{3}$$

With use of equation (1) and footnote 8

$$k'(T) = \frac{1}{hF(T)} \int_0^\infty \exp(-E/kT) \left[\int_0^{E-E_a} \rho^\ddagger(E - E_a - \epsilon)\, d\epsilon \right] dE \tag{4}$$

Now let us change the variables of integration to ϵ and E' according to the transformation

$$E' = E - E_a - \epsilon$$
$$\epsilon = \epsilon$$

The Jacobian of the transformation is unity, the range of integration now extends to infinity for both variables and we get:

$$k'(T) = \frac{\exp(-E_a/kT)}{hF(T)} \times \int_0^\infty \int_0^\infty \rho^\ddagger(E') \exp(-E'/kT) \exp(-\epsilon/kT)\, d\epsilon\, dE' \tag{5}$$

the integration over ϵ gives kT, the integration over E' gives the partition function of the activated complex $F^{\ddagger}(T)$ and we get finally the ordinary absolute reaction rate formula:

$$k'(T) = \frac{kT}{h} \frac{F^{\ddagger}(T)}{F(T)} \exp(-E_a/kT) \qquad (6)$$

2. Excitation of Molecules and the Creation of High Angular Momenta.—Molecules excited by electronic impact receive vibrational excitation due to the fact that the potential surfaces for the various electronic states do not have the same equilibrium separations and the Franck-Condon principle applies to the transitions. There is little or no rotational energy change for the molecule in transitions of this type since the impacting electron is light and in all considerations above it is assumed tacitly that the molecules have small rotational energy.

In contrast to this situation consider cases of excitation of molecular motion by recoil of one of the constituent atoms. The affected atom will not usually be at the center of mass of the molecule, and so states of high angular momentum are expected to result. During the decomposition of such a molecule as an isolated system, its angular momentum is conserved and the energy of rotation acts like an additional potential. Consequently the magnitudes of the various activation energies and the positions of the activated states depend upon the rotational state. In such cases it is necessary to use $k'_J(E)$, a rate constant for the energy E and the rotational state J, where by J we mean the complete designation of a particular rotational state.

The effect of rotational state on reaction rate constants has usually been neglected although it has been known to exist.[10] In most hot atom and molecule processes it must be considered.

3. Application to Collision Processes.—It is desirable to have a method for calculation of the probability that a chemical reaction occurs in an energetic collision, e.g., between a molecule (essentially at rest) and a "hot" atom or radical. The rate constant $k'(E)$ of equation (1) obviously does not apply to this case if taken as a bimolecular rate constant, since essentially all the energy is in translation. The initial state, therefore, is not described by a microcanonical ensemble of systems *randomly distributed* over all accessible states which have energy E.

In order to apply the theory described above let us consider two different chemical changes which can result from a given energetic collision. The ratio of the probabilities for occurrence of these two reactions will be given under certain conditions by

$$\frac{W^{\ddagger}{}_{1J}(E - E_{1J})}{W^{\ddagger}{}_{2J}(E - E_{2J})} \qquad (7)$$

where the $W^{\ddagger}$ factors for the activated states are defined above; E_{1J} and E_{2J} are the activation energies for the two reactions for the rotational state J. This ratio gives the ratio of the number of classical trajectories which have the correct constants of motion and complete the two reactions, respectively. This fact alone does not guarantee that ratio 7 will give the correct reaction product ratio, since the trajectories through the activated states are not necessarily continuations of the collision trajectory. Transfers of energy between various degrees of freedom are involved and these may not be rapid enough. If all other degrees of freedom of the system can transfer energy to the relative motion degree of freedom in the collision time, ratio 7 should give the correct product ratio.

We are led to suggest that the fraction of collisions of the system with energy E and rotational state J which lead to the ith reaction of a system is given by

$$f_{iJ}(E) = \frac{W^{\ddagger}{}_{iJ}(E - E_{iJ})}{\sum_j W^{\ddagger}{}_{jJ}(E - E_{jJ})} \qquad (8)$$

The summation must be extended over all possible reactions. One important process which is always possible is return to the original reactants. This process does not usually have an activation energy and an activated state is difficult to define precisely.

One application of this theory has been carried out and is described in the next section.

There is also interest in collisions which may have long duration (i.e., many oscillation periods of the complex) such as "sticky" collisions between ions and molecules,[11] or the recombination of free radicals.[9] In such cases the lifetime of the complex is of interest as well as the product distribution of the isolated complex since the ultimate products may actually depend upon whether the complex collides before it decomposes. The lifetime is determined by the decomposition rate which is given by the sum of terms $k'_i(E)$ (or $k'_{jJ}(E)$ if this treatment is needed). The product distribution for the isolated complex is, of course, also given in this case by equation (8).

4. The Hot Atom Reaction of Br^{80} with HBr.—Following the absorption of a thermal neutron in Br^{79} the Br^{80} atom which is formed receives a recoil due to the emission of γ radiation.[5] If all the energy of the nuclear process is released in one quantum, the energy given the ratioactive Br^{80} atom is approximately 400 electron volts. Neither the distribution of gamma energies or recoil energies is known[12] but it is certain that there are many "hot" bromine atoms formed. Kinetic studies with gaseous HBr have been made by Williams and Hamill[5] and a mechanism is suggested in which a "hot atom" exchange of Br^{80} with the Br atom of HBr is an important

step. According to these studies there is approximately a 25% chance that Br^{80} which starts with high kinetic energy will (in pure HBr) appear as HBr^{80} by the time that it is thermalized.

The author has used the ideas expressed in part 3 above in an attempt to calculate this exchange probability. Energetic $Br^{80}HBr$ complexes formed in collision can decompose in three ways.

$$Br^{80}HBr \rightarrow \begin{cases} Br^{80} + HBr & \text{original products} \quad (a) \\ HBr^{80} + Br & \text{exchange} \quad (b) \\ Br^{80}Br + H & \text{bromine formation} \quad (c) \end{cases}$$

We do not include dissociation into three atoms, which will occur predominantly at high energies, and therefore we apply a correction at high energies for dissociation of product molecules. The fraction of collisions at relative energy E for a particular rotational state which yield the exchange reaction is

$$f(E, J) = \frac{W^{\ddagger}_{bJ}(E - E_{bJ})p(E, J)}{W^{\ddagger}_{aJ}(E - E_{aJ}) + W^{\ddagger}_{bJ}(E - E_{bJ}) + W^{\ddagger}_{cJ}(E - E_{cJ})} \qquad (9)$$

Here $p(E, J)$ is the probability for forming an undissociated HBr^{80} molecule in the exchange reaction.

The $W^{\ddagger}$ functions were calculated with constants obtained from "semi-empirical" potential energy surfaces.[1] The exchange probability is most sensitive to the constants for the reactions (b) and (c); fortunately these are satisfactorily determined by the potential energy surfaces. Reaction (a), which yields the original products, has no activated state. Effectively a collision radius had to be assumed and it was arbitrarily taken to be the radius at which the average interaction energy of Br with the vibrationless HBr target molecule rises to 1 kcal./mole. The result is not extremely sensitive to this choice, since reaction (a) is several times faster than (b) and (c) combined. Furthermore, since the interaction potential rises steeply, a collision radius is fairly well determined.

In figure 1 results of the calculation are given. Here the probability for exchange is given as a function of the incident energy of Br^{80} atom, and an average over the various rotational states has been carried out, i.e.,

$$f(E) = \overline{f(E, J)} = \frac{1}{\Omega}\sum_{J} f(E, J) \qquad (10)$$

where Ω is the number of rotational states in which the collision complex can exist. A calculation was also made for DBr using the same potential surfaces and this is also given.

No calculation was actually made of the factor $p(E, J)$; it was taken to be unity throughout the indicated region, $0 \leq E \leq 16$ ev and zero for $E > 16$ ev. We note that the formation of dissociated HBr as a collision

product has its threshold at about 7.2 ev (since only half of the energy is in the center of mass system). Dissociation should be virtually complete when the average HBr product molecule receives 5.5 ev, since HBr cannot exist as a stable molecule with more energy.[13] Taking account of angular momentum conservation, the molecule shares the available energy (center of mass system) in two degrees of freedom with the relative translation. Thus, when two-thirds of the energy of 5.5 ev, or the total energy is about 8 ev, HBr should usually dissociate due to possession of excess energy. The corresponding incident energy of Br is about 16 ev.

The expression for $f(E)$ was averaged over the "cooling" process in order to calculate the total probability for exchange during thermalization.[13] The calculation yielded 25% in agreement with the experimental values

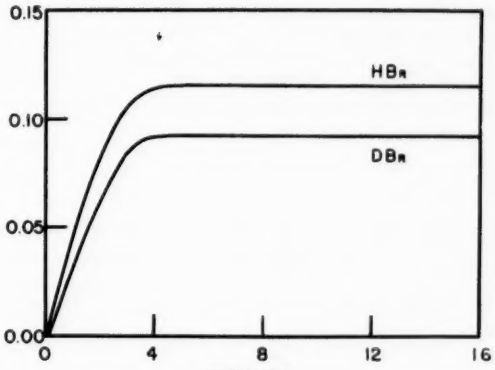

FIGURE 1

Probability for exchange reaction as a function of the incident atom energy in electron volts.

of Williams and Hamill.[5] For DBr a value of 21% was calculated; as yet there is no experimental work with which to compare this value.

Although the details of this particular calculation are not to be trusted, it is believed that a qualitatively correct description of the exchange process is obtained. The methods described here may be useful in other problems.

* A contribution from the Radiation Project, operated in part under Atomic Energy Commission Contract AT(11-1)-38.

[1] Eyring, H., (*a*) *J. Chem. Phys.*, **3**, 107 (1935); (*b*) Glasstone, S., Laidler, K. J., and Eyring, H., *Theory of Rate Processes*, McGraw-Hill, New York, 1941.

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[6] Rosenstock, H. M., Wallenstein, M. B., Wahrhaftig, A. L., and Eyring, H., PROC. NATL. ACAD. SCI., **38**, 667–678 (1952).

[7] Magee, J. L., Meeting of American Physical Society, Pittsburgh, April 1951. Notre Dame Radiation Project Report, 1950.

[8] If classical methods apply, the density function $\rho(E)$ for a system with N degrees of freedom can be obtained as $\left[\frac{\partial W(\epsilon)}{\partial \epsilon}\right]_{\epsilon=E}$, where $W(\epsilon) = \int \cdots \int \frac{dp_1 \cdots dp_N dq_1 \cdots dq_N}{h^N}$ and the integration is extended over all of phase space for which the energy is equal to or less than ϵ. The relation between the density function $\rho(E)$ for any system and its partition function $F(T)$ is

$$F(T) = \int_0^\infty \rho(E) \exp(-E/kT)\, dE$$

[9] Marcus, R. A., *J. Chem. Phys.*, **20**, 359 (1952). References to previous unimolecular decomposition work are given here.

[10] Reference 1(*b*), pp. 131, 260.

[11] Burton, M., and Magee, J. L., *J. Phys. Chem.* (in press).

[12] Yosim, S., and Davies, H., *Ibid.*, **56**, 599 (1952).

[13] More details of this work are available in a Notre Dame Radiation Project report.

*THE EFFECT OF AUXIN ON GROWTH AND RESPIRATION OF ARTICHOKE TISSUE**

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Communicated July 8, 1952

In the course of studies on a variety of storage tissues, Reinders[1] noticed that the uptake of water by disks of Jerusalem artichoke tubers (*Helianthus tuberosus*) was promoted by indoleacetic acid (IAA) "... to an exceedingly great extent, as has not occurred with any of the other objects." It was thought worth while, therefore, to use this tissue for experiments on the action of auxin, in parallel with our studies on potato tuber. In these it has been found (Hackett and Thimann[2]) that water uptake is promoted by naphthaleneacetic acid, but that indoleacetic acid has relatively little effect.† For this reason the behavior of the artichoke takes on added interest. In the present paper the observation of Reinders is substantiated, the effects of metabolic inhibitors on the auxin-induced water uptake have been determined, and the influence of auxin on the respiratory rate of the tissue has also been studied. In the artichoke, as in the potato, it is clear that the effects of auxin are dependent upon the aerobic metabolism of the tissue.

Materials and Methods.—Artichokes were purchased locally and then

stored briefly in a cold room (3 or 10°C.). Disks were prepared, washed and maintained aerobically on nets in the manner previously described for potato tissue (Hackett and Thimann[2]). The uptake of water was calculated directly from changes in blotted fresh weights. In no case did the tissues show signs of injury, nor did the solutions become turbid, even when water uptake was being strongly inhibited. Oxygen uptake measurements were made with replicate groups of 10 disks using constant volume Warburg respirometers (cf. Hackett and Thimann[3]).

Stock solutions of the inhibitors were prepared and the pH adjusted to 6.0–6.2, except in the case of 2,4-dinitrophenol, where the pH was 5.5 All solutions, unless otherwise stated, contained 10 mg./l. indoleacetic acid.

Results: The Effect of Auxin on Water Uptake.—The effect of IAA (10 mg./l.) on the time course of water absorption by artichoke disks over a 6-day period is shown in figure 1. This large promotion is even greater than that reported by Reinders.[1] The maximum effect is exerted during the first four days, and there is not the characteristic delay in response to auxin which was found with potato disks. Disks in IAA solutions are thicker, frequently curled at the edges, and very bumpy, suggesting a large amount of cell division. (It is perhaps significant that this tissue has been found very easy to bring into indefinite culture by Gautheret and his coworkers.) In twelve experiments, the mean value of the increase in fresh weight of disks in IAA for 4 days was 60 per cent, the absolute values ranging from 47 to 81 per cent; the corresponding increase in weight in pure water averaged only 6 per cent. Naphthaleneacetic acid (10 mg./l.) and 2,4-dichlorophenoxyacetic acid (1 mg./l.) gave comparable large promotions of the water uptake in one experiment. Three other samples of tubers behaved atypically both to auxin and to inhibitors and have been omitted from consideration.

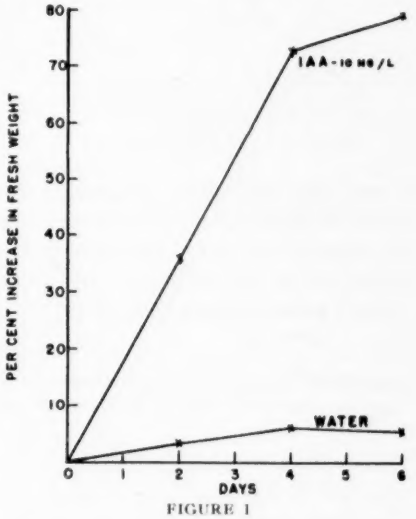

FIGURE 1

The time course of increase in fresh weight of artichoke disks in water and in indoleacetic acid (IAA, 10 mg./l.).

The Effects of Metabolic Inhibitors.—The effect of arsenite on the IAA-

induced water uptake during 4 days is shown in table 1. A duplicate experiment gave essentially the same results. It is clear that arsenite is a very effective inhibitor, the 50 per cent inhibiting concentration being slightly less than $1 \times 10^{-5} M$. At this and higher concentrations, arsenite caused the formation of a reddish or red-brown color in the normally white tissue.

TABLE 1

THE EFFECT OF ARSENITE ON THE IAA-INDUCED WATER UPTAKE BY ARTICHOKE TISSUE. EACH VALUE REPRESENTS THE MEAN FRESH WEIGHT IN GRAMS OF TWO SAMPLES, EACH CONTAINING 10 DISKS

SOLUTION	INITIAL WEIGHT	WEIGHT AFTER 4 DAYS	INCREASE, %
Water	1.165	1.23	5.6
IAA, 10 mg./l.	1.20	1.915	59.6
IAA + 3×10^{-5} *M* arsenite	1.16	1.76	51.8
IAA + 1×10^{-5} *M* arsenite	1.18	1.36	15.3
IAA + 3×10^{-6} *M* arsenite	1.19	1.27	6.7

The inhibiting effect of various concentrations of 2,4-dinitrophenol is shown in figure 2. The relationship between the increase in fresh weight, expressed as per cent of control after 4 days, and the logarithm of the concentration of DNP is linear. In two experiments, $3 \times 10^{-5} M$ DNP inhibited water uptake 49 and 39 per cent, respectively.

TABLE 2

THE EFFECT OF AZIDE AND FLUOROACETATE (FAc) ON THE IAA-INDUCED WATER UPTAKE BY ARTICHOKE TISSUE, AS MEASURED BY INCREASE IN FRESH WEIGHT AFTER 4 DAYS

SOLUTION	INCREASE IN FRESH WEIGHT, %	INCREASE AS PER CENT OF CONTROL
IAA, 10 mg./l.	47.7	..
IAA + 3×10^{-5} *M* azide	49.4	104
IAA + 1×10^{-4} *M* azide	22.2	47
IAA + 3×10^{-4} *M* azide	6.6	14
IAA + 3×10^{-4} *M* FAc	57.8	121
IAA + 1×10^{-3} *M* FAc	42.6	89
IAA + 3×10^{-3} *M* FAc	24.7	52

Table 2 includes data on the effect of azide and fluoroacetate on the water uptake. In this experiment the 50 per cent inhibiting concentrations were roughly $1 \times 10^{-4} M$ azide and $3 \times 10^{-3} M$ fluoroacetate. Iodoacetate was also found effective, the 50 per cent inhibiting concentration being roughly $3 \times 10^{-4} M$.

The effect of Auxin on Oxygen Uptake.—The time course of the oxygen uptake (Q_{O_2} = cu. mm. O_2 per hour per gram initial fresh weight) of disks

in water and in IAA, 10 mg./l., is shown in figure 3. The Q_{O_2} of disks in water falls off during the first two days to a low level, at which it remains for 4 days. This agrees with the earlier findings of Steward and Berry.[4] IAA causes an enormous stimulation of the oxygen uptake throughout the 6-day period. From the 2nd to the 6th day, the Q_{O_2} of disks in IAA is 400 per cent greater than that of those in water. This must be taken as very strong evidence that the effect of auxin is exerted primarily on oxidative metabolism.

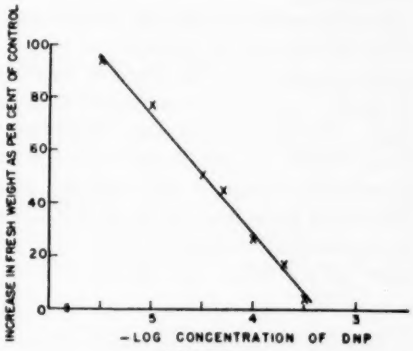

FIGURE 2

The increase in fresh weight, as percentage of that of controls in indoleacetic acid alone, of artichoke disks after 4 days in 2,4-dinitrophenol.

Discussion.—Since the sites of action of the inhibitors used above are known (see Hackett and Thimann[2]), certain conclusions may be drawn regarding the metabolic processes linked to the IAA-induced water uptake by artichoke disks. In general, it is clear that this process is dependent upon the aerobic metabolism. More specifically, the participation of —SH-containing enzymes, phosphorylation reactions, a heavy-metal oxidase and acetate metabolism is implicated. This has also been shown to be true for other effects of auxin. A quantitative comparison of the 50 per cent inhibiting concentrations for water uptake by potato and artichoke disks in the presence of auxin is given in table 3. These values show a striking agreement, indicating the fundamental identity of the processes involved. A comparison of these values with those giving comparable inhibitions of cell elongation by *Pisum* stems

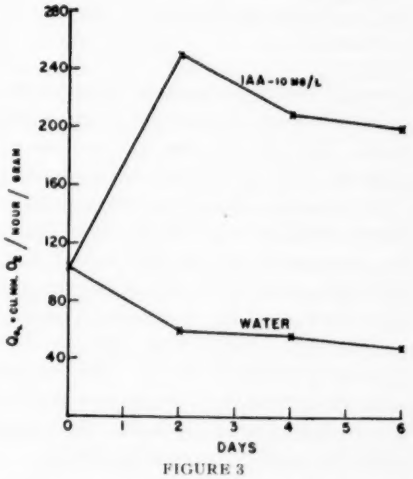

FIGURE 3

The time course of oxygen uptake (absolute Q_{O_2}) of artichoke disks in water and in indoleacetic acid (IAA, 10 mg./l.).

and *Avena* coleoptiles (cf. table 1 of Hackett and Thimann[2]) also points to the similarity between the action of auxin on water uptake and on cell elongation.

Further evidence linking the effect of auxin on water uptake with an effect on the aerobic metabolism is provided by the great stimulation of oxygen uptake. On the basis of measurements of changes in dry weight, Reinders[1] concluded that auxin stimulates the respiration of artichoke disks. Lachaux,[5] measuring the oxygen uptake of fragments of artichoke tissue cultured *in vitro*, found only a small promotion of the respiration by indoleacetic acid. In such cultures, the decrease in total sugars is also stimulated by auxin (Goris[6]) and this was attributed in part to the effect on respiration. In our experiments the oxygen uptake was increased more than 400 per cent. This effect of IAA is of a different order of magnitude

TABLE 3

COMPARISON OF CONCENTRATIONS OF INHIBITORS CAUSING 50% INHIBITION OF WATER UPTAKE BY POTATO AND ARTICHOKE DISKS

	CONCENTRATION $\times 10^{-5}$ *M*			
	ARSENITE	DINITRO-PHENOL	AZIDE	FLUORO-ACETATE
Potato disks in naphthaleneacetic acid (10 mg./l.)	3	3	7	100
Artichoke disks in indoleacetic acid (10 mg./l.)	1	3	10	300

from the responses shown by other tissues (cf. Smith[7]). Perhaps the nearest approach to it is given by tobacco pith. In this material, Newcombe[8] found that the ascorbic oxidase was increased four- to tenfold by IAA after 6–14 days, but the total respiration was increased barely 50 per cent.

It is worth noting that the stimulation of respiration is comparable in degree with the increase in water uptake. If this is expressed as a rate, i.e., increase in fresh weight per day, to compare more directly with respiration rates, we find that IAA increases the rate of water uptake (averaged over the 4 days) by a factor of 10 times.

Summary.—Indoleacetic acid causes a large increase in the rate of water uptake by artichoke tissue. This increase is accompanied by a comparable increase in the rate of respiration, which averages 400 per cent after 2 days. Azide, fluoroacetate, arsenite, dinitrophenol, and iodoacetate strongly inhibit the auxin-induced water uptake.

* This work was supported by a grant from the Committee on Growth acting for the American Cancer Society, Inc., to whom we are much indebted.

‡ Note added in proof: It has since been found that this difference is due in large part to bacterial destruction of indoleacetic acid. Sterile potato disks show a large promotion of water uptake by IAA.

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*ANALYSIS OF THE BIOCHEMICAL MECHANISM OF DRUG RESISTANCE IN CERTAIN BACTERIAL MUTANTS**

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Relatively little evidence is available on the biochemical mechanisms responsible for drug resistance (for references cf. 1, 2). An unusual opportunity to discriminate among certain possible mechanisms arose following the observation that a single compound, *p*-nitrobenzoic acid (PNB), could competitively interfere with the utilization of two different metabolites, *p*-aminobenzoic acid (PAB) and *p*-hydroxybenzoic acid (POB).[3] In other words, both PAB and POB, which have similar structures but separate metabolic functions, are required to reverse inhibition of growth by their common analog, PNB.

When strains resistant to each of these two inhibitions were compared with each other, and with strains resistant to inhibition by other analogs of PAB and POB, the results obtained provided evidence against a number of possible mechanisms of resistance. They were consistent, however, with another mechanism: a decrease in affinity of an enzyme for the drug. Further support for this mechanism was obtained by comparing the competitive ratio of drug to metabolite in drug-resistant mutants with the ratio in the more sensitive parent strain.

Cultural Methods.—The experiments were performed with the W strain of *E. coli* (ATCC 9637) in minimal medium A[4] supplemented with 100 μg. of DL-aspartic acid per milliliter. This supplement was added in order to prolong the inhibitory effect of the analogs of POB, and that of PNB acting as an analog of either POB or PAB; these inhibitions are otherwise quite transient.[3] Solid medium was prepared by the addition of 1.5% agar. Cultures were incubated at 35°C.

Isolation of Resistant Mutants.—Mutants of *E. coli* resistant to PNB were sought under two sets of circumstances. In the first of these, wild-

type cells were inoculated on a solid medium containing an inhibitory concentration of PNB (200 μg./ml.) plus sufficient PAB (10 μg./ml.) to reverse completely the anti-PAB action of this inhibitor. The organisms were still inhibited in this medium by the anti-POB action of PNB, but large inocula (10^8 to 10^9 cells) gave rise to a few colonies of mutant strains resistant to this inhibition. In a similar manner, mutants resistant to the anti-PAB action of PNB were isolated on a medium containing PNB (200 μg./ml.) plus POB (10 μg./ml.).

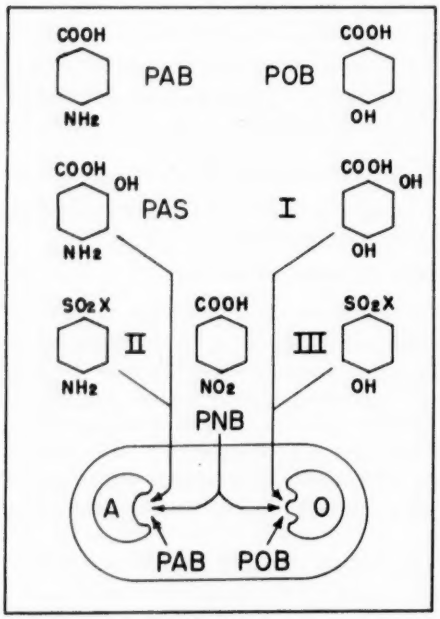

FIGURE 1

Schematic representation of competition by analogs of PAB and POB. I = β-resorcylic acid. II = sulfathiazole (X = 2-thiazolylamino) or diaminodiphenyl sulfone (X = p-aminophenyl). III = phenosulfazole (X = 2-thiazolylamino) or dihydroxydiphenyl sulfone (X = p-hydroxyphenyl).

Similarly, mutants resistant to inhibition by each of four other antimetabolites were selected: sulfathiazole and 4,4′-diaminodiphenyl sulfone, which compete with PAB; and the corresponding compounds with hydroxy instead of amino groups (phenosulfazole[5] and 4,4′-dihydroxydiphenyl sulfone), which have been shown to compete with POB.[3, 6] The structures of these compounds are shown in figure 1.

Specificity of Resistance.—The two types of PNB-resistant mutants were found to show essentially no cross-resistance: i.e., the type that was resistant to PNB plus PAB was not resistant to PNB plus POB, and vice versa. But decreased penetration of PNB would impede both its actions equally, and so could not produce separate resistance to each of these two actions. Increased destruction of the drug would likewise impede both its actions equally. These two possible mechanisms of resistance are therefore excluded in these strains.

Further comparisons were made with mutants resistant to the other analogs of PAB and POB mentioned above (table 1). Extensive cross-resistance was found between the sulfonamide and the sulfone analog of

TABLE 1

RESISTANCE OF VARIOUS STRAINS TO ANALOGS OF POB AND PAB[a]

STRAIN NUMBER →		WILD TYPE	PS-4	OS-2	NA-2	NO-1	ST-1	DA-1
RESISTANCE FOR WHICH SELECTED →			PHENOSULFAZOLE	DIHYDROXYDIPHENYL SULFONE	PNB ANTI-POB	PNB ANTI-PAB	SULFATHIAZOLE	DIAMINODIPHENYL SULFONE
ADDITION TO MEDIUM	CONCENTRATION, μG./ML.	GROWTH						
None	—	+	+	+	+	+	+	+
Phenosulfazole	200	−	+	+	−	−	−	−
Dihydroxydiphenyl sulfone	200	−	+	+	−	−	−	−
PNB	200	−	−	−	+	−	−	−
+ PAB	10							
PNB	200	−	−	−	−	+	−	−
+ POB	10							
PNB	200	−	−	−	−	−	−	−
Sulfathiazole	2	−	−	−	−	−	+	+
Diaminodiphenyl sulfone	10	−	−	−	−	−	+	+

[a] The strains listed were all streaked on a single plate of each medium; these plates were then incubated for 48 hours. Solid rather than liquid media were used in order to avoid the outgrowth of any resistant mutants that might arise during the experiment. The growth recorded is therefore that of the predominant population, occasional colonies being neglected.

PAB, as would be expected, and similarly between the two corresponding analogs of POB. There was little or no cross-resistance, however, between PNB as a competitor of PAB, and the sulfonamide and sulfone competitors of PAB; similarly, there was little or no cross-resistance between PNB as a competitor of POB, and the other competitors of POB.

Since in the sulfonamide or sulfone analogs of PAB or POB it is the substituent on carbon atom 4 of the metabolite that is replaced, whereas in PNB it is the substituent on carbon atom 1, it would be of interest to determine whether specific resistance could also be obtained to an analog resulting from substitution in yet another part of the molecule. Among such compounds, an analog of POB substituted at position 2, β-resorcylic acid

TABLE 2

CROSS-RESISTANCE AMONG PAB ANALOGS SUBSTITUTED AT CARBON 1[a]

STRAIN →		W		ST-1		DDS-1		PAP-1	
ADDITION	μG./ML.	GROWTH AT 1 AND 2 DAYS							
None	..	2	4	2	4	1/2	4	2	4
Sulfathiazole	0.1	1	4	2	4	1/2	4	1	4
	0.2	0	4	2	4	1/2	4	0	4
	0.5	0	0	2	4	*m*	4	0	0
	1.0	0	0	1/2	4	0	*m*	0	0
	2.0	0	0	0	0	0	0	0	0
Diaminodiphenyl sulfone	2	2	4	2	4	1/2	4	..	..
	5	0	4	2	4	1/2	4	..	..
	10	0	0	2	4	1/2	4	..	..
	20	0	0	1/2	4	*m*	4	..	..
	40	0	0	0	0	0	3	..	..
p-Aminophenylphosphonous acid	20	2	4	2	4	..	..	2	4
	50	1	4	2	4	..	..	2	4
	100	0	1/2	1	4	..	..	2	4
	200	0	0	0	4	..	..	1	4

[a] From the wild type (W), one-step mutants were selected for resistance to sulfathiazole (ST-1), diaminodiphenyl sulfone (DDS-1), and *p*-aminophenyl-phosphonous acid (PAP-1). About 100 cells of each strain, in 5 ml. of solid medium supplemented as indicated, were poured in small Petri dishes (5 cm. diam.). Growth was recorded after incubation: *m* = microscopic; 1 = barely visible; 4 = full growth.

(Fig. 1), was found at concentrations of 200–600 μg./ml. to be an effective competitor of POB in *E. coli*. Mutants resistant to β-resorcylic acid were accordingly isolated, and were found to show little cross-resistance to the other analogs of POB under investigation.

The corresponding analog of PAB, *p*-aminosalicylic acid (PAS) (Fig. 1), is known to compete with PAB in tubercle bacilli. We were unable to utilize PAS in the present study because this compound, even in high concentrations, failed to inhibit *E. coli*. However, while this work was in

progress Yegian and Long[7] showed that in tubercle bacilli there was no cross-resistance between PAS and sulfathiazole.

Attention was next directed to a comparison of analogs substituted at the same position rather than at different ones. It is well known that sulfonamide and sulfone analogs of PAB, which are substituted at position 1, show cross-resistance, as has been seen in table 1 with sulfathiazole and diaminodiphenyl sulfone. In table 2, however, which employs a more refined test, it is seen that cross-resistance between these two inhibitors is not complete; strain ST-1 is approximately twice as resistant as strain DDS-1 to sulfathiazole, and half as resistant to diaminodiphenyl sulfone.

A mutant resistant to another 1-substituted competitive analog of PAB, *p*-aminophenyl-phosphonous acid[8] (PAP) was also obtained. As is seen in table 2, the mutant resistant to PAP showed no cross-resistance to sulfathiazole, though the sulfathiazole-resistant mutant did show cross-resistance to PAP. Incomplete cross-resistance was also obtained between sulfathizole and another analog substituted at the same position, *p*-aminophenylarsonic acid (*p*-arsanilic acid, Atoxyl), but these results are not presented in detail.

Similar results have been obtained with two analogs of POB that are substituted at position 4. One of these, PNB, has been described above; the other, PAB, though required as a metabolite at low concentrations, is also at much higher concentrations a competitor of POB.[3] Mutants resistant to PAB, and those resistant to the anti-POB action of PNB, showed only partial cross-resistance.

Specificity of resistance to various inhibitors was originally observed with one-step mutants that were two to eight times as resistant as the parent strain; the differences persisted even after higher degrees of resistance had been obtained by successive selections.

It is clear that the mechanism of drug resistance can discriminate between different analogs even when the substitutent groups are as similar as a sulfonamide and a sulfone. The important feature of the above observations, for the present analysis, is this specificity, rather than the fact that in some cases some degree of cross-resistance does exist.

This specificity of resistance excludes a number of possible mechanisms, to be discussed below, which could not discriminate between different analogs of the same metabolite. It is compatible, however, with the hypothesis that these resistant mutants possess an enzyme with decreased affinity for the drug compared with its affinity for the competing metabolite.

Competitive Ratio of Analog to Metabolite (Inhibition Index).—This hypothesis of an altered enzyme was tested further on the basis of the expectation that a shift in the relative affinity of the enzyme for the drug and for the metabolite should be associated with a corresponding shift in the competitive ratio of these two substances for growing cells.

In this study, to insure that the endogenous metabolite formed would have no significant influence on the competitive ratio, two concentrations of the drug, both in excess, were used. Table 3 shows that at both these concentrations the competitive ratio of sulfathiazole to PAB was about 25 in the parent strain, 200 in a one-step sulfathiazole-resistant mutant, and 1000 in a multi-step resistant mutant; furthermore, these competitive ratios were proportionate to the levels of drug resistance of these three

TABLE 3

SULFATHIAZOLE SENSITIVITY, AND SULFATHIAZOLE/PAB COMPETITIVE RATIO, IN WILD TYPE AND IN RESISTANT MUTANTS[a]

STRAIN →		W	ST-1	ST-2
SULFATHIAZOLE, μG./ML.	PAB, μG./ML.	LIGHT TRANSMISSION, %		
0	0	50	50	56
0.2	0	51	...	...
0.3	0	68	...	...
0.5	0	99	...	...
2.0	0	...	52	...
3.0	0	...	94	...
5.0	0	...	100	...
10.0	0	...	...	58
15.0	0	...	...	72
20	0	...	...	92
40	0	100	100	100
40	0.015	...	...	95
40	0.02	...	...	92
40	0.03	...	...	83
40	0.05	...	...	67
40	0.15	...	85	...
40	0.2	..	63	...
40	0.3	...	53	...
40	1.0	92	...	...
40	1.5	71	...	...
40	2.0	52	...	...
120	0.05	...	...	99
120	0.10	...	...	88
120	0.15	...	...	80
120	0.2	...	...	81
120	0.3	...	99	78
120	0.5	...	80	...
120	0.7	...	51	...
120	3.0	90	...	...
120	5.0	65	...	...
120	7.0	52	...	...
Sulfathiazole sensitivity		0.3	2.5	15
ST/PAB ratio (ST = 40)		40/1.5 (27)	40/0.18 (210)	40/0.04 (1000)
ST/PAB ratio (ST = 120)		120/4.5 (27)	120/0.6 (200)	120/0.1 (1200)

[a] Strain ST-1 was derived from the wild type (W) by one selective exposure to sulfathiazole, and strain ST-2 in turn was similarly derived from ST-1. With each strain 10^{-4} ml. of a fresh 18-hr. culture, resuspended in water, was inoculated into 10 ml. of minimal medium A, supplemented as indicated, in tubes 22 mm. in diameter. After 24 hrs. of incubation, turbidity was measured with an Evelyn photoelectric colorimeter, filter 620 mμ.

Fifty per cent inhibition was taken as the end point for the interpolations at the bottom of the table. This value was equivalent to 70% transmission, except with strain ST-2 at 120 μg./ml. sulfathiazole, where it was equivalent to 88% transmission. (In this last case the very high drug concentration produces partial inhibition that was not reversed by PAB; only the PAB-reversible inhibition was considered.)

Essentially identical results were obtained with the pour-plate technique described in table 2, except that the partially inhibitory concentrations observed were about one-half those obtained in tubes.

strains. Likewise, in strains resistant to a POB competitor (phenosulfazole) a similar shift was observed in the competitive ratio of drug to metabolite. These results support the inference of an enzyme of altered affinity.

Sulfonamide Resistance in a PAB Auxotroph.—Further evidence against certain possible mechanisms was obtained by observing the effect of sulfonamide resistance on the PAB requirement of a mutant of *E. coli*. Starting with PAB auxotroph 48A-33, a resistant derivative was obtained by two successive selections with sulfathiazole. These selections were carried

TABLE 4

EFFECT OF SULFATHIAZOLE RESISTANCE ON PAB REQUIREMENT[a]

PAB, MμG./ML.	STRAIN 48A-33 (SENSITIVE)			STRAIN 48A-33ST2 (RESISTANT)		
	18 HRS.	24 HRS.	48 HRS.	18 HRS.	24 HRS.	48 HRS.
	LIGHT TRANSMISSION, %					
0	100	100	100	100	100	100
0.1	98.5	97	96	100	100	99
0.2	96	94.5	90	99	99	99
0.4	91	83	80	98.5	96.5	95
0.8	83	73.5	74	95	83	75
1.6	73.5	64	66	82	66	68
3.2	62.5	60	55	73	60	57
6.4	56.5	56	50	60	56.5	50
12.8	53	53.5	46	53	53.5	46

[a] Strain 48A-33, a stable PAB auxotroph, and strain 48A-33ST2, a sulfonamide-resistant strain derived from it, were each inoculated (5×10^{-3} ml.) into colorimeter tubes, incubated, and measured turbidimetrically as in table 3.

Essentially identical results were obtained with streaks on solid medium.

out in a medium containing a PAB concentration (0.01 μg./ml.) that could satisfy the growth requirement of the bacteria but would not reverse the inhibitory effect of the drug at the concentrations used. The derivative strain was found to be 15 times as sulfathiazole-resistant as its parent strain.

Table 4 shows that this resistant derivative not only still requires PAB, but actually has an increased threshold of response. With an excess of

PAB it responds as well as its parent, but with decreasing concentrations of PAB it grows more slowly than its parent, and at very low concentrations (0.1–0.4 mμg./ml.) the resistant strain not only grows more slowly, but also yields much less final growth.

The significance of these results will be discussed below.

It has similarly been observed that β-alanine auxotrophs retain their requirement following mutation to resistance to an inhibitory competitor of β-alanine (D-serine).[9]

Discussion.—The following possible changes in resistant mutants suggest themselves as mechanisms that might account for the resistance.

1. Decreased penetration of the drug.
2. Increased destruction of the drug (or decreased conversion of an inactive to an active compound).
3. Increased concentration of a metabolite antagonizing the drug.
4. Increased concentration of an enzyme utilizing this metabolite.
5. Decreased quantitative requirement for a product of the metabolite.
6. Alternative metabolic pathway by-passing the metabolite.
7. Enzyme with decreased relative affinity for the drug compared with the metabolite.

As has been pointed out above, both mechanisms 1 and 2 (decreased penetration and increased destruction of the drug) have been excluded as explanations of PNB resistance in the mutants studied, since these mutants show that resistance to each of the two inhibitory actions of PNB (competition with PAB and with POB) develops independently.

In interpreting further observations, it is assumed that PAB is the substrate of only one enzyme and POB that of another, and that the several analogs of each substrate compete with it at the active site of its enzyme (Fig. 1). If this assumption is correct, the specific resistances observed with different competitors of the same metabolite eliminate mechanisms 3 to 5; for these mechanisms would involve only quantitative alterations in the substrate-enzyme-product system, and such changes could not discriminate between two different competitors of the same metabolite. Mechanism 6 (an alternative metabolic pathway) would also be unable to discriminate between these competitors, and would in addition be difficult to reconcile with the observation that resistance is graded rather than all-or-none. Finally, the persistence of a PAB requirement in a sulfonamide-resistant derivative of a PAB auxotroph likewise excludes mechanisms 3, 5 and 6.

All these observations, however, are compatible with mechanism 7: production of an enzyme with decreased affinity for the inhibitor compared with its affinity for the substrate. But the inference of this mechanism would require not only the assumption given in the preceding paragraph,

but also two further assumptions: (*a*) that the exclusion of mechanisms 1 and 2 can be extended from PNB to the other, structurally similar analogs; and (*b*) that no other possible mechanisms have been overlooked. Though these three assumptions seem reasonable, they cannot be experimentally tested at this time. Mechanism 7 therefore lacks rigorous proof.

Further, more direct evidence for this mechanism is provided by the demonstration that resistant strains have an altered competitive ratio of analog to metabolite, proportionate to their degree of resistance. A similar shift in competitive ratio was previously observed by Ivanovics in sulfonamide-resistant strains of staphylococci.[10] But even this evidence is not conclusive since it would also be compatible with mechanisms 1, 2, 4 or 5. However, the value of this evidence is strengthened by the existence of reasons for excluding these mechanisms.

The shift in competitive ratio was interpreted by Ivanovics as evidence for an enzyme with increased affinity for PAB.[11] This mechanism, however, could not distinguish between various analogs of PAB, and hence could not account for the specificity of resistance that we have observed. Moreover, the threshold PAB requirement of a PAB auxotroph was found to increase rather than decrease following development of sulfathiazole resistance. These observations suggest that in the resistant mutants there is a decrease in affinity of the enzyme for the metabolite, together with an even greater decrease in affinity for the drug.

This apparent decrease in affinity for the metabolite suggests that in this species optimal PAB utilization might be necessarily associated with sensitivity to sulfonamides. On this basis it would be easy to understand why a sulfonamide-sensitive wild type should have greater evolutionary survival value, in the ordinary sulfonamide-free surroundings, than the more sulfonamide-resistant mutants to which it frequently gives rise.

We have been led to postulate that mutations can lead to a variety of changes in the affinity of an enzyme for different drugs; this picture would imply the production of a variety of changes in the configuration of the binding site of the enzyme. This possibility is more readily visualized when it is noted that the competitors with the most strikingly differing resistance are those that differ in the positions of their substituent groups: PAB or POB analogs substituted at position 1 (carboxyl) of the ring, compared with respective analogs substituted at positions 2 or 4. It is of interest to note, however, that some specificity of resistance was also observed with different analogs substituted at the same position.

That enzymes of identical function in different species can differ in their relative affinity for various compounds is well known from observations of comparative biochemistry. For example, such enzymes have been shown, after isolation in the crystalline state, to differ in immunological specificity[12] and in susceptibility to various inhibitors;[13] and hemoglobins

found in the animal kingdom exhibit a 200-fold range in the ratio of their affinities for oxygen and for carbon monoxide.[14] The results described in this paper strongly suggest that such a change in affinity can result from a single mutation. This inference is further strengthed by the direct demonstration with extracted enzymes, reported elsewhere,[15] that a mutation can cause bacteria to produce an enzyme altered in another quality, temperature sensitivity.

A more rigorous test for an enzyme of altered affinity would be possible if this enzyme could also be studied in extracts of the cells, but the enzymes involved in the present study have not seemed promising for such investigation. Evidence for altered dehydrogenase systems in extracts has been reported by Sevag and Gots,[16] who studied pneumococci resistant to a number of drugs that appear to interfere with riboflavin metabolism; but it has not been established that inhibition of these dehydrogenases is the mechanism by which these drugs interfere with growth.

It is not implied that the mechanism inferred here is the only one responsible for drug resistance. Indeed, an example of mechanism 2 is the formation of penicillinase by many penicillin-resistant organisms; and an example of mechanism 3 is increased PAB production, which has been reported for certain sulfonamide-reistant bacteria. However, even in these sulfonamide-resistant strains other mechanisms may also be involved, since the degree of resistance is not always paralleled by the amount of PAB excreted.[17]

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Summary.—*p*-Nitrobenzoic acid is an inhibitory competitor of both *p*-aminobenzoic acid and *p*-hydroxybenzoic acid in *E. coli*. Mutants resistant to either of these inhibitions are not resistant to the other. These resistances could therefore not be due to decreased penetration or increased destruction of the drug.

Resistance to various analogs of a single metabolite has also been observed to be partly or completely specific. This finding excludes a number of other possible mechanisms of drug resistance.

These results are all compatible with the presence of an altered enzyme, with decreased relative affinity for the inhibitor compared with the metabolite. This hypothesis is supported by the observation that increased resistance is accompanied by a proportionate increase in the competitive ratio of analog to metabolite. In addition, evidence is presented that in some resistant strains the PAB-utilizing enzyme has a decreased affinity for PAB, as well as an even more decreased affinity for the sulfonamide analog.

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PRODUCTION OF AN ALTERED PANTOTHENATE-SYNTHESIZING ENZYME BY A TEMPERATURE-SENSITIVE MUTANT OF ESCHERICHIA COLI

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Studies on the growth requirements of many microbial mutants have shown that in each of these strains a single biosynthetic reaction is blocked. To account for these blocks it has been proposed that a mutant either (*a*) fails to produce any enzyme protein corresponding to the blocked reaction;[1] or (*b*) produces an altered, inactive protein;[1] or (*c*) produces an inhibitor of a normal enzyme.[2]

A number of attempts have been made to test these hypotheses, mainly with mutants of Neurospora,[2–5] including a temperature-sensitive one.[6] In several of these studies one of these mechanisms was ruled out but no definitive evidence for either of the remaining mechanisms was obtained. In the present study, however, it was possible to demonstrate one of the three mechanisms in a temperature-sensitive auxotroph of *Escherichia coli*.

This mutant requires pantothenate only above 30°C.; below this temperature it grows, like the wild type, without pantothenate. It was de-

derived from an ordinary auxotroph which requires pantothenate at any temperature. For both strains pantoate and β-alanine are inactive as growth factors, indicating a block in the last stage of pantothenate synthesis.

To test the above three mechanisms, extracts of the temperature-sensitive auxotroph and of the wild type were studied with respect to the enzymatic coupling of β-alanine and pantoate to form pantothenate. The first stage of this study, extraction of the pantothenate-synthesizing enzyme from the wild type and its characterization in cell-free extracts, has already been reported.[7, 8] In the present paper wild-type extracts will be compared with those of the mutant; it will be shown that in the latter an altered, extraordinarily heat-labile pantothenate-synthesizing enzyme is present.

Strains.—Mutant 99-1, which requires pantothenate at any temperature, was obtained from the W strain of *E. coli* (ATCC 9637) by means of the penicillin technique.[9] Mutant 99-1t, a temperature-sensitive pantothenate auxotroph, was isolated as a secondary mutant from strain 99-1 after plating a heavy inoculum of the latter into minimal medium A[10] and incubating at 25°C. Under these conditions only prototrophs and temperature-sensitive pantothenate auxotrophs grew out. The latter, which comprised 5 to 10 per cent of the colonies tested, were distinguished from the prototrophs by their inability to grow on minimal medium A at 37°C.

Extraction of the Enzyme.—All strains were grown in medium A enriched with yeast extract and casein hydrolysate (NY Medium).[11] The enzyme was obtained in cell-free solution by extracting acetone-dried cells, or fresh cells that had been ground with glass. The former procedure has been outlined previously.[8] Although it is less laborious than grinding fresh cells it yielded preparations that varied greatly in their enzyme activity. This was especially true of extracts of mutant 99-1t, a number of which were found to be completely inactive. For most of the experiments to be described in the present paper, therefore, the extracts were obtained from ground fresh cells.

For this procedure the cultures, after having reached maximal turbidity, were harvested in a Sharples centrifuge, and were washed twice with distilled water and collected into a pellet by centrifugation. The cell mass was ground in a mullite mortar in the cold with twice its weight of powdered Pyrex glass and was suspended in M/15 potassium phosphate buffer at pH 7.0 for one hour at 5°C. Suspended matter was removed by centrifugation for one hour at 14,000 r. p. m. at 5°C. with the high-speed attachment of the refrigerated International Centrifuge. As with extracts from acetone-dried cells,[8] all of the enzyme was shown to remain in solution. The extract from either the temperature-sensitive or the normal strain could be stored in the frozen state at −10°C. for at least two weeks without loss of activity.

Measurement of Enzyme Activity.—Experiments will be reported on the enzyme activity of extracts and of non-growing cell suspensions. With

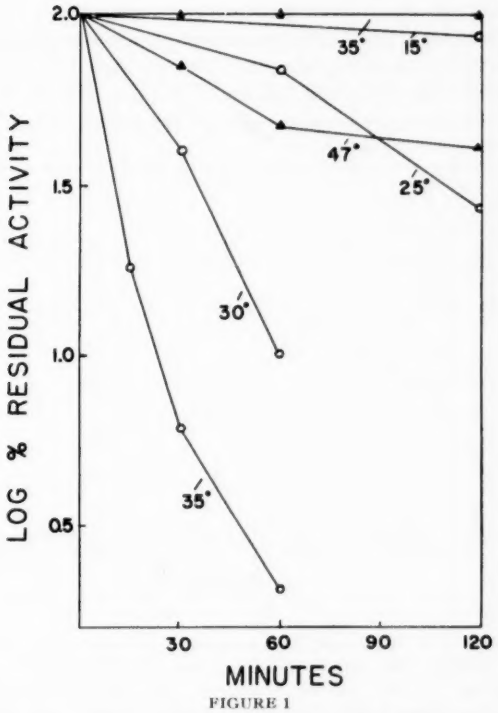

FIGURE 1

Effect of temperature on the stability of the pantothenate-synthesizing enzyme in extracts of mutant 99-1t and of the wild type. Acetone-powder extracts of the two strains were incubated at the temperatures noted. At the times indicated samples were removed, cooled to 15°C. and tested for enzymatic activity. In these tests, each tube received extract from 40 mg. of acetone powder of the mutant or extract from 3 mg. of acetone powder of the wild type. In addition, the testing tubes contained in mMolar concentrations: β-alanine 20, K pantoate 20, K_2ATP 10, KCl 100, $MgSO_4$ 10, tris-(hydroxymethyl)-aminomethane (Tris) buffer pH 8.5 100; total volume 1.0 ml. After incubation for one hour samples were assayed for pantothenate as described in the text. 100% residual activity equals 86 mμ moles of pantothenate per ml. per hour for 99-1t, 178 mμ moles of pantothenate per ml. per hour for the wild type. O-O- = mutant 99-1t; ▲-▲- = wild type.

the extracts, activity was measured in the presence of β-alanine, pantoate, adenosine triphosphate (ATP), and appropriate cationic activators.[8] After incubation samples were diluted with distilled water at 80°C. to a

level suitable for assay and were kept at 80°C. for 5 minutes to inactivate the enzyme. Assays for pantothenate were performed with mutant 99-1 as described previously;[11] several dilutions of each sample were assayed, at least two of which were within the sensitive range.

To measure activity of cell suspensions, cells were washed twice with distilled water by centrifugation and then incubated in phosphate buffer with β-alanine, panotate and glucose.[11] In this system ATP could not replace glucose as energy source. After incubation samples were centri-

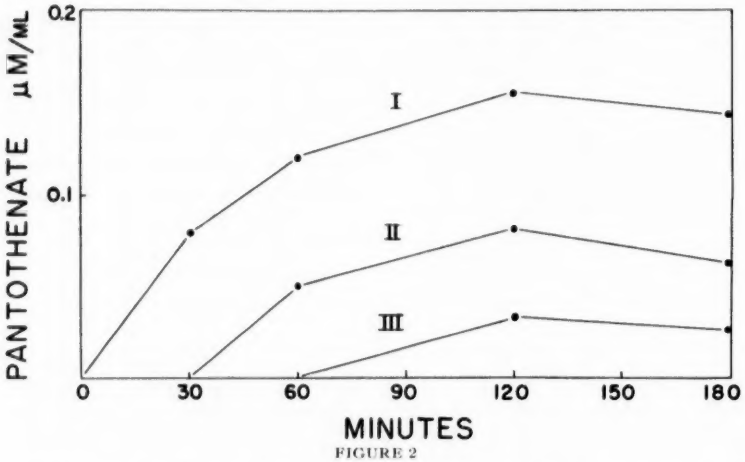

FIGURE 2

Inactivation at 27°C. of mutant 99-1t enzyme in presence and absence of the factors required for activity. The acetone-powder extract, prepared in the cold from a suspension of 40 mg. of powder per ml., was divided into three equal portions (I, II, III). The first portion (I) was incubated at 27°C. with the factors required for activity. The other two (II, III) were kept at 27°C. for 30 and 60 minutes, respectively, before addition of the other factors, and then were further incubated at the same temperature. The added factors were (mMolar): β-alanine 20, K pantoate 20, K_2ATP 10, KCl 100, $MgSO_4$ 10, Tris buffer pH 8.5 100; total volume 1.0 ml. Pantothenate was determined by assay at the times indicated.

fuged to remove cells and then heated and assayed for pantothenate as in the extract experiments.

Heat-Lability of the Enzyme in Extracts of the Temperature-Sensitive Mutant.—To study the stability of the enzyme, extracts of mutant 99-1t and of the wild type were incubated, without additions, at various temperatures. At intervals samples were removed and cooled, and their residual enzyme activity was determined at 15°C. by incubation with the required factors. This low temperature was necessary because at higher temperatures the enzyme in the mutant extract is inactivated appreciably

during the 30- to 60-minute testing period. At 15°C., on the other hand, the mutant enzyme maintains a constant rate of pantothenate synthesis for at least three hours, showing that at this temperature it is stable. Furthermore, since a constant rate of enzyme activity was observed with extracts heat-inactivated to a varying extent, it is clear that the enzyme is

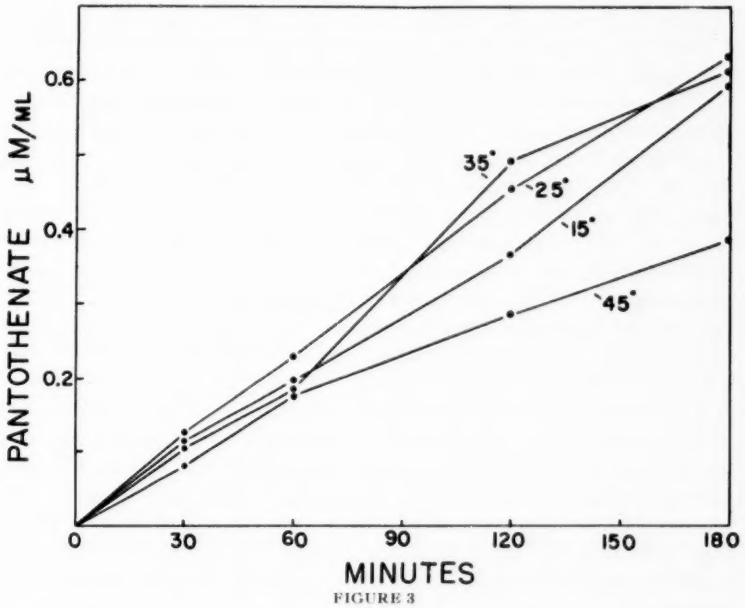

FIGURE 3

Effect of temperature on the activity of the wild-type enzyme. The concentration of the extract was reduced threefold for each 10° rise in temperature in order to maintain the same small per cent conversion of the substrates in all tubes. The tube incubated at 15°C. contained extract from 3 mg. of acetone powder. In addition, each tube received a mMolar concentration: β-alanine 20, K pantoate 20, K_2ATP 10, KCl 100, $MgSO_4$ 10, Tris buffer pH 8.5 100; total volume 1.0 ml. Pantothenate was determined by assay at the times indicated.

not reactivated at 15°C. after having been inactivated at higher temperatures.

One such experiment on the heat-lability of the enzyme is shown in figure 1. The enzyme in the extract of the temperature-sensitive mutant is seen to differ strikingly from that in the wild-type extract. Whereas the latter is completely stable for two hours at 35°C. and is inactivated only slightly at 47°C. during that period, the former is largely inactivated within one hour at 30°C.

In the experiment described in figure 1 the stability of the enzyme was tested in the absence of the factors required for enzyme activity. The presence of these factors, however, does not significantly change the heat stability of the enzyme of either strain. For the mutant a comparison between resting and actively synthesizing enzyme is shown in figure 2. For the wild type, the data for the actively synthesizing enzyme are presented in figure 3; those for the resting enzyme have been presented in figure 1.

It should be noted in figure 2 that in the time interval between 120 and 180 minutes, when the pantothenate-synthesizing enzyme had been largely inactivated, there is actually a slight decrease in pantothenate concentration. This decrease is due to the presence in the extracts of another enzyme, which slowly removes pantothenate from the solution. As has been described,[8] this enzyme can be inhibited by a pantothenate analog, tolyl pantothenone, without impairing the activity of the pantothenate-synthesizing enzyme. The activity of the pantothenate-removing enzyme is usually insignificant in comparison with that of the pantothenate-synthesizing enzyme. Thus, when the experiment described in figure 1 was repeated in the presence of tolyl pantothenone, at a concentration that prevented pantothenate disappearance, essentially the same rates of enzyme inactivation were observed.

The heat stability of the mutant enzyme varies somewhat from one preparation to the next. For instance, at the same temperature the enzyme preparation described in figure 2 was inactivated more rapidly than that described in figure 1. In general, mutant extracts prepared from wet cells are more stable than those from acetone powders. It should be pointed out, however, that variations in heat stability among mutant extracts are small in comparison with the differences between mutant and wild-type extracts.

The heat stability of the enzyme from either the wild type or the mutant was found to be unaffected by the temperature at which the strains were grown; this factor did affect, however, the yield of enzyme from the temperature-sensitive mutant. For the experiment described in figure 1, both the mutant and the wild-type strain were grown at 15°C. and without aeration. These conditions were suitable for obtaining a good yield of enzyme from the mutant. Mutant cells grown anaerobically at 25°C. yielded considerably less enzyme, while aerobic conditions at 25°, or growth at 35°, resulted in completely inactive preparations. Extracts of the wild type, on the other hand, were equally active whether grown anaerobically at 15°C. or aerobically at 35°C.

Evidence that the Mutant Enzyme Itself Is Excessively Heat-Labile.—The results presented so far suggest that mutant 99-1t produces a more heat-labile enzyme protein than the wild type. However, the possibility has not been eliminated that the observed difference in enzyme stability might

be due to differences in factors other than the enzyme molecules. This hypothesis could be tested experimentally. If the two extracts are mixed, the presence in the mutant extract of a heat-activated enzyme inhibitor, or of a factor that sensitized the enzyme to heat inactivation, would be expected to decrease the thermal stability of both enzymes; conversely, the presence of a stabilizer in the wild-type extract would be expected to stabilize both enzymes. Inactivation rates were therefore studied at 35°C. in mixtures of mutant and wild-type extracts.

TABLE 1

PROTECTIVE EFFECT OF SUGARS AND NEUTRAL SALTS ON HEAT INACTIVATION OF THE TEMPERATURE-SENSITIVE MUTANT ENZYME

PROTECTIVE AGENT	EXPOSURE TO 35°C. 1 HR.	2 HRS.	24 HRS.
	% INACTIVATION		
None	84	96	100
Sucrose 50%	0	0	37
Sucrose 25%	47	..	..
Maltose 50%	0	..	..
Maltose 25%	27	..	..
KCl 20%	0	..	..
KCl 2.5%	61	..	..

Glass-ground extracts (0.2-ml. portions) were incubated at 35°C. in the concentrations of the substances indicated for the periods noted. After incubation the extracts were cooled to 15°C. and residual enzyme activity was determined as in figure 1. Each tube contained, in addition to the factors required for activity, extract from 5 mg. of cells (dry weight).

TABLE 2

HEAT INACTIVATION OF THE PANTOTHENATE-SYNTHESIZING ENZYME IN A MIXTURE OF WILD-TYPE AND TEMPERATURE-SENSITIVE MUTANT EXTRACTS

EXTRACT		EXPOSURE TO 35°C. 0 MIN.	30 MIN.	60 MIN.
WILD-TYPE	99-1t	RESIDUAL ENZYME ACTIVITY (PANTOTHENATE mμ MOLES/ML./HR.)		
+	−	155	133	151
−	+	182	27	5
+	+	338	169	164

Glass-ground extracts (0.1-ml. portions) of each strain were incubated in small test tubes, alone or in mixtures, at 35°C. for the periods noted. Wild-type extract contained material extracted from 2 mg. of cells (dry weight) per 0.1 ml. of solution, 99-1t extract contained that from 5 mg. of cells per 0.1 ml. After incubation at 35° the tubes were cooled to 15°, the factors required for enzyme activity were added and residual enzyme activity was determined as in figure 1.

A typical experiment, carried out like that described in figure 1, is presented in table 1. It can be seen that after exposure to an elevated tem-

perature the residual activity of the mixture, determined at 15°C., is equal to the sum of the activities of the separate controls, indicating that the rate of heat inactivation of the enzyme in either extract is not affected by the presence of the other extract. Similar results have been obtained with mixtures of the two extracts in other proportions. It is concluded that the observed difference in thermal stability of the extracts arises from a difference in the enzyme molecules themselves.

This conclusion should be qualified by the reservation that either extract might contain a substance that affected the stability of the enzyme, but was tightly bound to it and not present in excess. Evidence against this possibility was furnished by the observations that dialysis against buffer did not affect the thermal stability of either enzyme; and addition to mutant extract of heat-inactivated wild-type extract (57°C. for 30 minutes), which might be expected to contain a stabilizer liberated from the inactivated enzyme, did not affect the thermal stability of the mutant enzyme.

The following observations lend further support to the conclusion that the mutant enzyme itself is excessively heat labile. As is seen in figure 1, the logarithm of the residual activity of this enzyme decreases linearly with time, indicating a unimolecular process, such as has generally been observed in heat denaturation of proteins In accord with this observation, the time required to produce a given per cent inactivation has been found to be independent of the initial enzyme concentration. The activation energy of the inactivation (Arrhenius constant), determined from the temperature dependence of its velocity constant, was found to be approximately 50,000 cal./mole (25° to 30°C. = 57,000 cal./mole; 30° to 35°C. = 49,000 cal./mole)—a value typical for protein denaturations. Furthermore, agents known to protect proteins from denaturation, such as sugars and neutral salts in high concentrations, also retarded inactivation of the mutant enzyme (table 2). In addition to the substances listed in table 2, reducing agents such as cysteine and cyanide at 0.5 *M* were found to afford some protection to the mutant enzyme (50% retardation of inactivation at 35°C). The above protective agents also prevented heat inactivation of the enzyme in the wild-type extract at 47°C. but were only slightly effective at 57°C.

Other Differences Between Extracts of the Wild Type and the Temperature-Sensitive Mutant.— In addition to differing in thermal stability the extracts of the two strains show slight differences in various other respects. Thus the Michaelis constant for pantoate in wild-type extracts (2.0 m*M*) is several times greater than that in mutant extracts (0.5 m*M*). The Michaelis constant for β-alanine, on the other hand, is the same in both extracts (0.5 *mM*). Also, the apparent affinity of the mutant enzyme for ATP is somewhat greater than that of the wild-type enzyme; this difference, however, may be due to a difference in enzymes other than the pantothenate-synthesizing one, since either extract hydrolyzes ATP at an appreciable

rate even in the absence of factors required for pantothenate synthesis. In regard to qualitative and quantitative dependences on activating ions the two extracts behave alike.

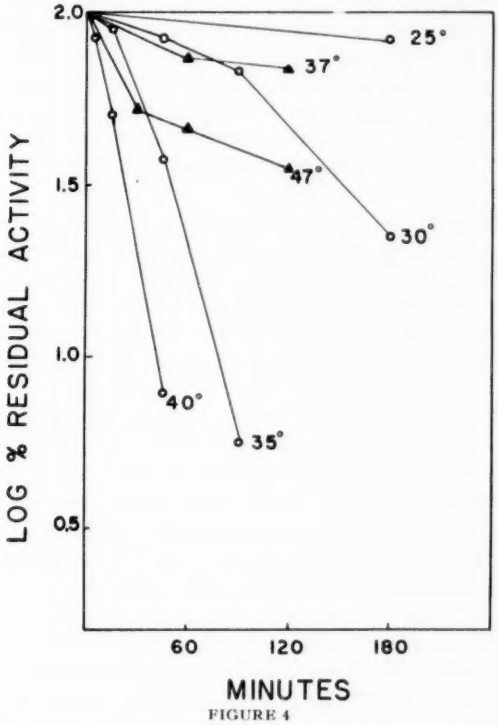

FIGURE 4

Effect of temperature on the stability of the pantothenate-synthesizing enzyme in resting cell suspensions of mutant 99-1t and of the wild type. Washed cell suspensions of the two strains were incubated in distilled water at the temperatures noted. At the times indicated samples were removed and tested for enzymatic activity at 25°C. In these tests an amount of mutant cells equivalent to 6 mg. dry weight of cells, and wild-type cells equivalent to 15 mg. dry weight, were used per tube. In addition, the tubes contained in mMolar concentrations: β-alanine 5, K pantoate 5, glucose 84, K phosphate buffer pH 7.0 100; total volume 1.0 ml. After incubation for one hour samples were assayed for pantothenate as described in the text. For 99-1t, 100% residual activity equals 9 mμ moles of pantothenate per ml. per hour; for the wild type 30 mμ moles per ml. per hour. O-O- = mutant 99-1t; ▲-▲- = wild type.

Urea, a denaturing agent, was found to be more effective in inhibiting mutant than wild-type enzyme. Furthermore, as mentioned before, acetone treatment yielded inactive preparations more often from the

mutant than from the wild type, suggesting that the mutant enzyme is more susceptible to destruction by acetone than the wild-type enzyme.

Heat Lability of the Enzyme in Non-Growing Cell Suspensions of the Temperature-Sensitive Mutant.—It was of interest to study the heat lability of the pantothenate-synthesizing enzyme in whole cells, for purposes of comparison with its lability in extracts. These experiments were carried out in a manner similar to that described in figure 1. The cells were incubated without any supplements in phosphate buffer at elevated temperatures, and at intervals samples were cooled and tested for enzyme activity at 25°C. In cell suspensions both enzymes are quite stable at that temperature.

Figure 4 shows that in cell suspensions, as has previously been shown in extracts, the mutant enzyme is considerably less stable than the wild-type enzyme. Comparison with figure 1, however, shows that the mutant enzyme is more stable in cell suspensions than in extracts: for a given inactivation rate, the suspensions require a temperature 5 degrees higher than the extracts do. The wild-type enzyme, in contrast, is no more stable in cell suspensions than in extracts.

In previous abstracts[12, 13] it was reported that inactivation of the mutant enzyme in the cells at 35°C. was markedly accelerated by the addition of glucose or other energy sources, although at 25°C. its stability was not affected by these substances. At that time, in the absence of information on the extracted enzyme, it was concluded that the observed temperature sensitivity of the enzyme was due to the action of an inhibitor, produced as a result of metabolic activity of the cell. However, in the light of the experiments on extracts it has become clear that the enzyme itself is heat-labile. The temperature sensitivity of the enzyme observed in whole cells therefore cannot be due to an inhibitor alone, but results from both the primary lability of the enzyme and the secondary effects of unknown intracellular factors.

This sensitivity of the enzyme in the cells can account for the heat sensitivity of the growth requirement, since the temperature at which the enzyme becomes unstable in cell suspensions coincides with that at which these cells lose the ability to grow without pantothenate. At 25° the mutant grows without pantothenate at the same rate as the wild type; up to 30° its growth becomes progressively slower; and above that temperature it does not grow at all without pantothenate.

Tests for Enzyme Activity in the Ordinary Pantothenate Auxotroph.—Extracts and non-growing cell suspensions of strain 99-1, which requires pantothenate at any temperature, were also studied. Details of these studies will be reported separately. In brief, though the cells were grown and extracted under a variety of conditions, no enzyme activity could be detected in either extracts or whole cells; the assay would have detected 0.0005 times that found in wild-type preparations.

Discussion.—The results obtained with a temperature-sensitive mutant show that mutation can result in the production of a qualitatively changed enzyme. Such genic control of the nature of an enzyme has already been suggested by studies on another type of protein, hemoglobin, which was found to be altered as a result of mutation to sickle-cell anemia in man.[14] Furthermore, evidence is presented elsewhere that mutation to drug resistance in bacteria can involve production of an enzyme altered in its affinity for the drug.[15]

Although a change in the concentration of an inhibitor or a stabilizer might have accounted for the heat lability of the mutant extract, this possibility was largely eliminated by experiments on mixtures of mutant and wild-type extracts. Such mixture experiments, however, could not exclude the possibility of an inhibitor or a stabilizer that was tightly bound to its enzyme. But it was possible in mixtures to inactivate all of the mutant enzyme and at the same time none of the wild-type enzyme; this fact would imply that any modifying substance in one of these extracts was all bound to its enzyme molecules, and affected all the enzyme molecules present in that extract. Such a stoichiometric attachment of a modifying factor to an enzyme could hardly be distinguished operationally from a change in the enzyme molecule proper.

It is of interest that the stability of the mutant enzyme is influenced by various factors in its environment. Thus, certain antidenaturing agents were found to retard its heat inactivation. In this connection it should be noted that the mutant enzyme is more resistant to heat inactivation in whole cells than in extracts, and that the heat inactivation in cells is accelerated by the addition of an energy source. The mechanisms by which these intracellular effects are produced are obscure.

The results presented here provide direct evidence for a one to one relationship between gene and enzyme. It should be pointed out that the one gene-one enzyme hypothesis, as originally stated by Beadle,[16] encompassed the possibility that a mutation could result not only in a quantitative change in an enzyme (including complete absence), but also in a qualitative change. The present experiments demonstrate for the first time such a qualitative change.

This demonstration strengthens the suggestion, made by Wagner and Haddox,[17] that a qualitatively changed enzyme might be the explanation for the behavior of an auxotroph of Neurospora which at first appeared to be an exception to the one gene-one enzyme hypothesis. (This mutant, despite a pantothenate requirement under most conditions, exhibited pantothenate-synthesizing activity in acetone-dried mycelium.) Furthermore, the present demonstration lends support to the possibility that ordinary auxotrophs, such as mutant 99-1, which apparently lack the enzyme, may nevertheless produce a protein that corresponds to the mutated gene.

It would be of interest to see whether or not the two mutations described in this paper are allelic. Other mutant pairs, of which both members are blocked in the same reaction, but one is temperature-sensitive, have been described in Neurospora; and in each of these pairs investigated the two mutations were found to be allelic.[18] Unfortunately, the W strain of *E. coli* does not recombine genetically. Studies on this problem are in progress, however, with mutants of the K-12 strain of *E. coli*, which can recombine.

In addition to shedding light on the problem of gene-enzyme relationships, the results reported have a bearing on the problem of temperature tolerance of micro-organisms. Bacterial species differ widely in the temperature ranges in which they are able to grow. In the present case, a decrease in temperature tolerance, produced in a microbial strain by a mutation, has been shown to be due to the presence of an enzyme of decreased thermal stability. Parallel observations have recently been reported with naturally occurring strains: several enzymes extracted from a thermophilic bacterium were found to be much more resistant to heat denaturation than the corresponding enzymes from closely related mesophilic bacteria.[19, 20]

Summary.—The enzyme that catalyzes pantothenate synthesis from β-alanine and pantoate has been studied in a temperature-sensitive mutant of *E. coli* which requires pantothenate for growth only above 30°C. When grown at low temperatures this mutant exhibited enzyme activity in both resting-cell suspensions and extracts; but the enzyme in either of these preparations was much more heat-labile than that in the corresponding preparations of the wild type. In mixtures of wild-type and mutant extracts, neither the thermal stability nor the activity of the mutant extract was affected by the presence of the wild-type extract, and vice versa. It is concluded that the mutation has resulted in the production of an altered, excessively heat-labile pantothenate-synthesizing enzyme. The implications of this finding for the problem of gene action are discussed.

Acknowledgment.—It is a pleasure to acknowledge the excellent technical assistance of Margaret C. Sanderson.

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*TRANSDUCTION OF MULTIPLE NUTRITIONAL REQUIREMENTS IN SALMONELLA TYPHIMURIUM**

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Recent studies reported by Lederberg, *et al.*,[1] at the 1951 Cold Spring Harbor Symposium on "Genes and Mutations" have demonstrated a system of genetic exchange in Salmonella which they have called "transduction." This system involves a filterable agent "FA" with mutagenic properties which is liberated from certain strains of Salmonella. The filterable agent is capable of transferring individual traits from a donor strain to other susceptible strains, even though the living cells are absent from the filtrate. Lederberg and Zinder state that individual filtrates may transduce a number of different traits, but no more than one to a single bacterium. Their studies involve various phenotypic characters including metabolic, drug-resistant, and antigenic variants.

The study involved in this paper confirms with independent strains that biochemical requirements of one Salmonella strain can be transduced singly, and in addition gives evidence that, in combinations involving several different nutritional requirements in each strain, multiple transductions can occur from the filtrate of the donor strain to a susceptible recipient strain. Not only the auxotrophic requirements, but also the wild type alleles present in the donor strain apparently may be transferred in this manner, since they appear in the recipient strain progeny treated with "FA" much more frequently than they do as reversions in isolations from the recipient strain alone. These transductions do not show the balanced reciprocal classes determined by chromosomal exchange in cases of recombination and crossing-over in sexually reproducing forms, but appear to be at random in no predictable proportions from one experiment to another.

Three strains of Salmonella have been used in our experiments, all

independent of those used by Zinder. Two of these are *Salmonella typhimurium:* No. 533 derived from Dr. J. W. Gowen's stock 11c, and No. 549 from Dr. P. R. Edwards. The third, No. 541, is *Salmonella schleissheim* obtained from Dr. F. Kaufmann of Copenhagen, Denmark. The multiple auxotrophic strains used in the tests reported here were obtained by successive x-ray or ultraviolet radiation exposures of the wild type 533 and 549 (cf. Plough, Young and Grimm[2]). For example, after x-radiation of suspensions of No. 549, 549-59 was obtained which required threonine for growth. The latter was subjected to further radiation and gave rise among others to 549-59-19 requiring proline in addition to threonine. A third x-radiation exposure produced two additional requirements in one strain, namely 549-59-19-1 requiring threonine, proline, isoleucine and valine. Multiple auxotrophs were obtained in this manner for several strains of *Salmonella newport* (511) and *Salmonella schleissheim* (541) as well as for the other strain of *Salmonella typhimurium* (533). The high frequencies of spontaneous reversions from multiple stocks of the two former species restricted us to the use of the *S. typhimurium* multiples, of which there are a number giving very low rates of reversion over a long period of time. Before using any multiple strain in a test of transduction the normal reversion rate was always determined as a preliminary precaution. The only multiples used were those which showed no growth in a $^1/_{10}$ dilution of a known saline cell suspension on synthetic medium from which at least one nutrilite required by the multiple had been omitted.

Experimental Procedures.—Two such stable strains, designated A and B, are used, having completely different multiple nutritional requirements. These strains are grown separately in flasks containing 60 ml. of Difco Brain Heart Infusion broth. After 18–24 hrs. incubation, each broth is filtered through a fine grade Seitz filter. A 3-ml. portion of each filtrate is incubated to test the sterility of the filtrate. Fifty ml. of each sterile filtrate are placed in separate flasks. B filtrate is inoculated with 1 ml. of an actively growing broth culture of A strain, and vice versa. As controls, one flask containing 50 ml. of BHI broth is inoculated with 1 ml. of strain A and another flask with strain B. In order to compare the effect of growing strains A and B together, a fifth flask containing 50 ml. of broth is inoculated with 0.5 ml. of each strain. The five flasks are incubated at 37°C. on a mechanical shaker. At 24- and 48-hr. intervals, 5 ml. are removed from each flask, centrifuged at 5500 r. p. m. for 20 minutes twice, each time being resuspended in 5 ml. of sterile distilled water. After making a $^1/_{10}$ dilution, 1-ml. aliquots of each sample are placed in separate plates which have been layered with MacLeod's minimal agar containing essential salts and glucose. To each one of these plates is added warm MacLeod's agar containing different combinations of nutrilites which should allow only transduced forms to grow. After thorough mixing, these plates are incubated for 48 hrs. and

then read. When colonies are present, a hundred or more are isolated and tested further for biochemical requirements according to the scheme in table 1.

TABLE 1

SCHEME USED FOR TESTING ISOLATES FOR TRANSDUCTION OF NUTRITIONAL REQUIREMENTS

Strain A requires R, S, T (R−, S−, T−, X+, Y+, Z+)
Strain B requires X, Y, Z (R+, S+, T+, X−, Y−, Z−)

SUPPLEMENTS ADDED TO MacL.	R S X Y	R S X Z	R S Y Z	S T X Y	S T X Z	S T Y Z	R T X Y	R T X Z	R T Y Z	None
Description of flask										
A										
B										
A + B										
A in B filtrate										
B in A filtrate										

Results.—The first indication of transduction found in this laboratory followed the use of the filtrate of 533-1159, one of the few multiples which acquired a triple amino acid requirement following only one dose of x-radiation. Radiation of 533, the wild-type *Salmonella typhimurium*, produced 533-1159 requiring arginine, methionine and aspartic acid. It was found that when 459-59-19-1 (described above), requiring threonine, proline, isoleucine and valine, was grown either mixed with or in the cell-free filtrate of 533-1159, strains were isolated with many different new combinations of the requirements of the parental strains. The reverse situation did not occur, no new types being found when 533-1159 was grown in the filtrate of 549-59-19-1. This particular combination was repeated three times with comparable results each time. The results of the second test are to be found in table 2.

One of the isolates from the above experiment designated $H_1 10(62)$ requiring methionine, aspartic acid and proline (marked with asterisk in table 2) was tested among others with 533-480-96-88, a multiple mutant requiring leucine, threonine and tryptophan. This strain was derived from wild type 533 which had been treated with ultra-violet to obtain a leucine auxotroph 533-480. Two subsequent treatments with ultra-violet added requirements for threonine and tryptophan, respectively. It was found that 533-480-96-88 was susceptible to the activity of "FA" from $H_1 10(62)$ but that a filtrate of 533-480-96-88 did not cause any changes in $H_1 10(62)$. See table 3.

Except for the prototrophs—not yet completely retested—many more transduced forms appeared following the growth of the susceptible strain in an active filtrate than when the two strains were grown together. The

TABLE 2

EXPERIMENTS WITH 533-1159 AS F.A.

Summary of transduced forms isolated from plates inoculated with washed cell suspension derived from BHI broth in which 549-59-19-1 and 533-1159 were grown together, and from plates inoculated with washed cell suspension obtained after 541-59-19-1 had grown in the sterile filtrate of 533-1159

Strain requirements: 549-59-19-1 requires threonine (T), proline (P), isoleucine (I) and valine (V). 533-1159 requires arginine (A), methionine (M), and aspartic acid As)

A	M	AS	T	P	I	V	NO. ISOLATED FROM 549-59-19-1 MIXED AND 533-1159	NO. ISOLATED FROM 549-59-19-1 IN 533-1159 FILTRATE
+	+	+	+	+	+	+	4	0
+	+	+	+	+	+	−	2	0
+	+	+	+	+	−	−	2	0
+	+	+	+	−	+	−	2	0
+	+	+	−	+	+	−	3	0
+	+	+	+	−	−	−	4	0
+	+	+	−	+	−	−	1	5
+	+	−	−	+	+	+	1	0
+	−	+	+	+	+	−	1	0
+	−	+	+	−	+	+	1	1
−	−	+	+	−	+	+	0	16
+	−	−	+	−	+	+	2*	0
+	−	+	+	−	+	−	4	0
+	−	+	−	−	+	+	0	1
−	+	+	−	−	+	+	0	1
−	−	+	−	−	+	+	0	11
−	−	+	−	+	+	−	0	1
−	+	+	−	+	−	−	0	1
+	−	+	−	+	−	−	1	2
+	−	+	+	−	−	−	3	0
+	−	+	−	−	−	+	0	1
+	−	+	−	−	+	−	0	1
−	−	+	−	+	−	−	0	4
−	+	−	−	+	−	−	0	1
+	−	−	−	−	+	−	1	0
+	−	−	−	−	−	−	2	0
−	−	−	−	+	−	−	0	2
+	−	+	−	−	−	−	1	1
−	−	+	−	−	−	−	0	1
−	−	−	−	−	−	−	1	0
−	−	+	−	+	−	−	0	1
−	−	+	−	+	−	−	0	2
−	−	−	−	−	−	−	0	2
							36	55

* is used to designate the wild-type alleles.

− is used to denote a requirement for the nutrilite under which it appears.

new types are numerous enough in test plates inoculated with cells grown in active filtrate so that only a small proportion of the total number are isolated. In contrast, the number of colonies in each plate of selective media inoculated with cells from mixtures usually gave only a small number of transduced forms (cf. table 1).

TABLE 3

Summary of nutrilite requirements of all strains isolated from plates inoculated with washed cell suspension derived from B.H.I. broth in which 533-480-96-88 and $H_1 10$ (62) were grown together, and from plates inoculated with washed cell suspension obtained after 533-480-96-88 had grown in the sterile filtrate of $H_1 10(62)$

Strain requirements: 533-480-96-88: Leucine, Threonine, Tryptophan. $H_1 10(62)$: Methionine, Aspartic acid, Proline.

L	T	Tr	M	As	P	NO. ISOLATIONS FROM MIXED CELLS	NO. ISOLATIONS FROM FILTRATE TREATED CELLS
+	+	+	+	+	+	63	3
−	−	−	−	−	−	1	0
−	+	−	+	+	+	2	1
−	−	−	+	+	+	1	0
−	+	+	+	+	−	0	25
−	+	−	−	+	+	1	0
−	+	−	+	−	+	0	1
−	+	−	+	+	−	1	3
−	−	+	+	+	−	0	1
−	+	+	+	−	−	0	2
−	−	+	+	−	−	0	1
−	−	+	−	+	−	0	1
−	+	−	−	−	+	1	0
−	−	−	+	+	−	2	0
−	+	+	−	−	−	0	1
−	−	+	−	−	−	0	1
−	−	−	−	+	−	2	0
−	−	−	+	−	−	1	0
+	+	−	+	+	−	0	5
+	−	−	+	+	−	1	0
+	+	−	−	+	−	1	0
+	+	−	+	−	−	1	0
+	+	−	−	−	−	1	1
+	−	−	−	−	−	3	1
+	−	−	+	−	−	1	0
						83	47

Shortcomings of the Method Used.—It is necessary to realize the shortcomings of the method described when used to analyze cases of transduction. For simplicity, following table 1, we shall call R−S−T− the requirements of the recipient cells, and X−Y−Z− the requirements of the

donor strain being tested. First, the media used do not test for all combinations of nutritional requirements, so that certain combinations will not be isolated. For instance, the following will not be isolated: (*a*) new types having minus alleles of all the auxotrophs (R−S−T−X−Y−Z−), and (*b*) those which have all the nutritional requirements of the recipient strain in addition to one or more of the donor cells as (R−S−T−X−Y+ Z+) and vice versa (R+S+T−X−Y−Z−). The labor involved in making all tests needed has been prohibitive up to the present. However, these types (*a*) and (*b*) do grow occasionally on the selective media used for reasons as yet unexplained, and therefore we know they do appear although not in proportion to the other types. In mixed suspensions outgrowth of an occasional colony of a strain requiring more nutrilites than are supplied in any particular selective medium is probably due to the satellite effect.[3] Second, some parent type colonies manage to grow on the selective media for the same reason. Third, the method does not show what proportion

TABLE 4

STRAINS SHOWING TRANSDUCTION

The first strain is transduced by the filtrate of the second strain listed.

	STRAINS	REQUIREMENTS
I	549-59-19-1	Threonine, proline, isoleucine, valine
	533-1159	Arginine, methionine, aspartic acid
II	533-321-1-2	Histidine, leucine, isoleucine
	Aj[1](52)[a]	Arginine, methionine, proline
III	533-480-96-88	Threonine, tryptophan, leucine
	Aj[1](52)[a]	Arginine, methionine, proline
IV	533-480-96-88	Leucine, threonine, tryptophan
	$H_1$10(62)[a]	Methionine, aspartic acid, proline

[a] Strains isolated from combination I.

of the total number of organisms in the suspension show transduction. Until a more precise method of identifying and isolating all of the transduced strains is devised, the method serves as a qualitative index of transduction rather than a quantitative one.

Discussion.—The data in tables 2 and 3 show that the filtrate of certain specific strains of Salmonella may cause transduction of one or several auxotrophic requirements in either the minus or plus direction in a susceptible recipient strain. These transduced requirements are individually determined, but one to six different ones may be produced in a single bacterium. They appear to be distributed at random among the recipient cells. Thus when the filtrate of one donor strain L+T+Tr+M−As−P− (table 3) acts on a recipient strain, L−T−Tr−M+As+P+, the isolates show a range in requirements from prototrophs having all wild type alleles, to multiple auxotrophs requiring all the amino acids represented. Many

of the intermediate types are found to be present but in no constant proportions. It may be noted in table 3 that in addition to the prototrophs, at least one transduced form appeared more often than the others, L−T+Tr+M+As+P−, but the reciprocal combination is absent. A count of the total number of transductions at each gene locus shows clearly that the mutant genes of 543-480-96-88 are more likely to revert to wild type under the influence of FA of $H_1$10(62) than are wild type genes to auxotrophs.

The ability to cause transduction in certain recipient strains appears to be restricted to a small number of donor strains of Salmonella, just as was found by Lederberg and Zinder. Strain 533-1159 and certain strains formed by transduction of FA from 533-1159 are the most effective agents in further transduction. Table 4 lists the combinations which have shown positive results.

In each case the second of the two strains listed in the combinations in table 4 is the transducing (donor) agent. In addition the transducing strains are selective in their action. Although the filtrate of 533-1159 was shown to cause transduction in 549-59-19-1, it has been tested with other strains on which it had no effect. Also $H_1$10(62) which is capable of causing transduction in 533-480-96-88 cannot cause any changes in strain 533-321-1-2. Thus transduction does not result when the filtrate of a donor strain is used with every other strain tested. The mechanism by which these effects are produced is unknown at present.

Summary.—Isolations from mixtures of Salmonella strains having different sets of auxotrophic requirements showed that interchange of some of the requirements had apparently occurred. When certain of the original strains were grown in the cell-free filtrates of cultures of other multiple strains it was found that requirements of the latter were transferred to the former, thus confirming independently the "transduction" of Lederberg and Zinder. The chief difference between our results and theirs is that several reqirements can be transferred at once to a single organism rather than one at a time.

* This research was aided by a contract with the Atomic Energy Commission, Division of Biology and Medicine. AT(30-1)-930.

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A COMPARISON OF THE EFFECTS OF X-RAYS AND THERMAL NEUTRONS ON DORMANT SEEDS OF BARLEY*

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Communicated by Donald F. Jones, July 10, 1952

Introduction.—The present investigation was initiated to compare the effects of irradiating dormant seeds of barley with x-rays and thermal neutrons on seedling height and chromosomal aberration frequencies. The results presented here show that there are differences in the response of dormant seeds of barley to x-rays and thermal neutrons. It is hoped that these data may be of some significance in interpreting the mechanism of the effect of ionizing radiations on biological systems.

No attempt will be made to review the literature relevant to the effects of x-rays and thermal neutrons on living organisms. The reader is referred to Lea[1] for a discussion of x-ray effects. The excellent paper by Conger and Giles[2], and papers by Frolik and Morris,[3, 4] Rasch,[5] Schmidt and Frolik[6] and Zirkle[7] constitute most of the available literature on the effects of thermal neutron radiation of living organisms.

Material and Methods.—In the present investigation samples of 250 dormant seeds of Himalaya barley, which had been kept in a humidity-controlled desiccator for at least three weeks prior to treatment, were subjected to the following dosages of irradiations at the Brookhaven National Laboratory: x-rays—5000 r; 10,000 r; 15,000 r; and 20,000 r; and thermal neutrons—3, 6, 9 and 12 hrs. at 4.65×10^8 N_{th}/cm.^{2}/sec.

For x-raying, the seeds were placed in a single layer in a Pyrex Petri dish located 21 cm. from the tube. The radiation was filtered with 1 mm. of aluminum and was administered at 250 kvp. and 30 ma. Under these conditions the seeds received approximately 1200 r per minute as determined with a Victoreen intergron.

For treatment with thermal neutrons the seeds were placed in a single layer in a leucite container and exposed in the thermal column of the nuclear reactor. Flux calculations were determined with gold foils exposed with the seed.

After irradiation, seeds from each treatment along with control samples were handled as follows: (1) 150 seeds of each treatment were planted in randomized blocks in greenhouse flats for observations on seeding height, and (2) 100 seeds were germinated in Petri dishes and root tips removed for cytological analyses.

Individual seedling height measurements were taken at 14 days after planting on the material grown in the greenhouse. The data obtained were subjected to an analysis of variance.

For the cytological analysis root tips were removed from germinating seedlings at the time of the first cycle of cell divisions. They were fixed and stored in Carnoy's 6:3:1 (alcohol:chloroform:acetic acid) solution. Anaphase bridge and fragment frequencies were determined on these samples, using the aceto carmine smear technique.

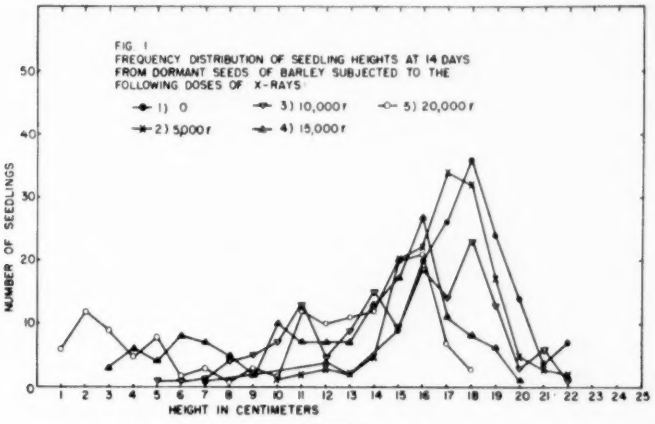
FIG. I
FREQUENCY DISTRIBUTION OF SEEDLING HEIGHTS AT 14 DAYS FROM DORMANT SEEDS OF BARLEY SUBJECTED TO THE FOLLOWING DOSES OF X-RAYS:
1) 0
2) 5,000 r
3) 10,000 r
4) 15,000 r
5) 20,000 r
NUMBER OF SEEDLINGS
HEIGHT IN CENTIMETERS

FIGURE 1

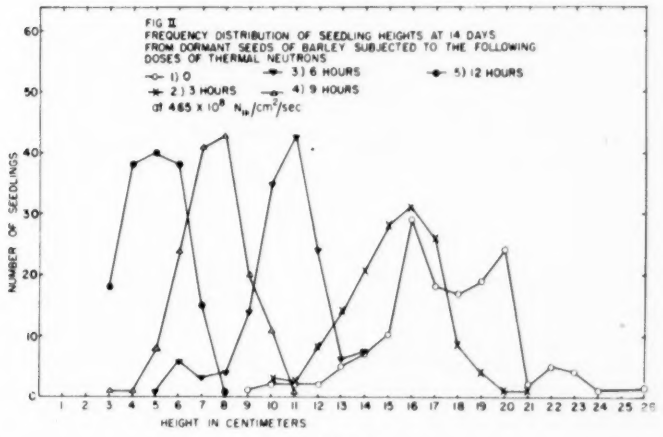
FIG II
FREQUENCY DISTRIBUTION OF SEEDLING HEIGHTS AT 14 DAYS FROM DORMANT SEEDS OF BARLEY SUBJECTED TO THE FOLLOWING DOSES OF THERMAL NEUTRONS
1) 0
2) 3 HOURS
3) 6 HOURS
4) 9 HOURS
5) 12 HOURS
at 4.85 x 10⁸ N_th/cm²/sec
NUMBER OF SEEDLINGS
HEIGHT IN CENTIMETERS

FIGURE 2

Experimental Results.—A striking difference was observed in the effects of x-rays and thermal neutrons on the height of seedlings at 14 days. With x-rays, at doses of 10,000 r and above, there was a considerably

greater range in individual seedling heights than there was with thermal neutrons. Furthermore, the magnitude of the difference increased with the increase in dosage of x-rays, so that at a dosage of 20,000 r individual seedling heights ranged from 1 to 18 cm. This has added significance when it is considered that the average height for the untreated control was only 17.7 cm. The frequency distributions of the seedling heights from seeds treated with x-radiation are depicted graphically in figure 1. According to an analysis of variance differences between treatments were significant.

When seeds were irradiated with thermal neutrons, individual seedling heights at any given dosage were no more variable than were the controls. Frequency distribution curves for the different dosages of thermal neutrons are presented in figure 2.

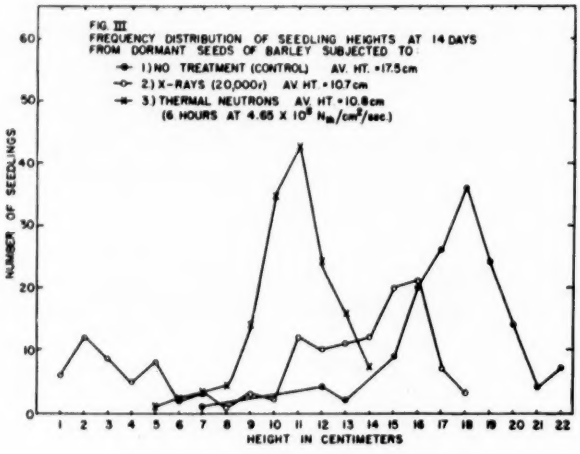
FIG. III
FREQUENCY DISTRIBUTION OF SEEDLING HEIGHTS AT 14 DAYS FROM DORMANT SEEDS OF BARLEY SUBJECTED TO
1) NO TREATMENT (CONTROL) AV. HT. = 17.5 cm
2) X-RAYS (20,000r) AV. HT. = 10.7 cm
3) THERMAL NEUTRONS AV. HT. = 10.8 cm
(6 HOURS AT 4.65 X 10⁸ Nth/cm²/sec.)
NUMBER OF SEEDLINGS
HEIGHT IN CENTIMETERS

FIGURE 3

In figure 3 frequency distributions are shown graphically from data which include a control along with a dosage of x-rays (20,000 r) and of thermal neutrons (6 hrs. at 4.65 $\times$ 10^8 N_{th}/cm.^{2}/sec.). These two irradiated lots gave about the same average decrease in seedling height. This serves to exemplify the different response of dormant barley seeds to these two types of radiations.

The treated material was permitted to continue growing in the greenhouse for 40 days following planting. At the end of that time differential killing of seedlings was apparent in the treatments receiving the three highest doses of x-rays. However, in the material irradiated with thermal neutrons, growth had ceased rather uniformly throughout the highest

dosage (12 hrs.). At all other doses of thermal neutrons, growth was continuing and there was no evidence of any killing.

Chromatinic bridges and fragments in anaphase cells from root tips of irradiated dormant seeds were used to estimate the chromosomal damage resulting from the irradiations. Data from the various treatments used are presented in tables 1 and 2. The highest dosage of x-rays resulted in a bridge and fragment frequency which was about the same as that obtained with a 3-hr. thermal neutron treatment. The highest dosage of thermal neutrons resulted in a bridge frequency of 1.5 per cell, which is about twice as great as has been noted previously with a lethal x-ray dose of 32,000 r (unpublished results).

TABLE 1

THE EFFECT OF THERMAL NEUTRONS ON FREQUENCIES OF BRIDGES AND FRAGMENTS IN TREATED DORMANT SEEDS OF BARLEY (ALL SEEDS WERE SUBJECTED TO A FLUX OF 4.65×10^8 N_{th}/CM.^{2}/SEC. FOR THE TIMES DESIGNATED)

TREATMENT	CELLS OBSERVED, NO.	BRIDGES PER CELL, NO.	FRAGMENTS PER CELL, NO.	ABNORMAL CELLS,[a] %
Control	114	0	0	0
3 hrs.	102	0.48	1.4	46
6 hrs.	88	0.79	1.8	65
9 hrs.	93	1.23	3.1	81
12 hrs.	96	1.53	4.6	92

[a] Cells with one or more bridges or fragments were considered abnormal.

TABLE 2

THE EFFECT OF X-RADIATION ON THE FREQUENCIES OF CHROMOSOMAL BRIDGES AND FRAGMENTS IN TREATED DORMANT SEEDS OF BARLEY (ALL DOSES GIVEN AT 250 KVP. AND 30 MA.)

TREATMENT	CELLS OBSERVED, NO.	BRIDGES PER CELL, NO.	FRAGMENTS PER CELL, NO.	ABNORMAL CELLS,[a] %
Control	102	0	0	0
5,000 r	402	0.13	0.33	19.6
10,000 r	494	0.30	1.1	34.2
15,000 r	139	0.37	1.3	41.6
20,000 r	106	0.48	1.4	50.3

[a] Cells with one or more bridges or fragments were considered abnormal.

Discussion.—Heretofore, studies comparing the effects of x-rays and thermal neutrons have indicated that these two types of radiations produce comparable biological effects, although their physical properties are vastly different. To the writers' knowledge this is the first demonstration that these two radiations may produce biologically different reponses. This is most evident in the distribution of individual seedling heights following irradiation of seeds with x-rays and thermal neutrons (see Figs. 1–3).

These observations show that individual barley seeds subjected to

identical doses of x-rays may be variously affected, whereas with identical doses of thermal neutrons they are injured much more uniformly.

When seeds are subjected to x-rays, events causing ionizations should be randomly distributed. With thermal neutrons this is not the case because some atomic nuclei have markedly higher cross sections for this type of radiation than do others. Thus, it seems possible that similar nuclear events have a greater chance of occurring equally in seeds subjected to thermal neutrons than in seeds subjected to x-rays. This may partially explain the seedling injury patterns reported here.

For years radio-biologists have been accustomed to obtaining differential survival of any class of organisms subjected to sufficient x-radiation to induce some killing. The reasons for this variation have been attributed to diverse factors including both genetical and physiological phenomena. With irradiated dormant seeds the writers had previously been of the opinion that the chance accumulation of chromosomally aberrant cells was a prime factor in differential killing. This is now considered unlikely, because with thermal neutrons it has been possible to obtain much higher aberration frequencies than was possible with x-rays before any killing took place.

For equal numbers of chromosomal aberrations thermal neutrons caused less seedling injury than x-rays. If this observation is interpreted on a cellular basis, it indicates that a considerable amount of x-ray-induced injury must be due to ionizations in extra-chromosomal elements of the cell. This is not intended to imply that thermal neutrons do not have some effect on such extra-chromosomal elements. However, it indicates that, in comparison with x-rays, proportionately more events occur in the chromosomal than in the extra-chromosomal elements of the cell.

If one assumes that ionization in either the chromosomal or extra-chromosomal constituents of the cell may be manifested in reduced growth, and that the two may be additive, the data for the x-ray-treated seeds are more readily understandable. Due to chance, either chromosomal or extra-chromosomal constituents could be affected in varying degrees and this could account for the divergent seedling heights obtained from seeds given the same dosage of x-rays.

Summary.—Dormant seeds of Himalaya barley were subjected to various doses of either x-rays or thermal neutrons. Seedlings from seeds treated with x-rays had a wide range in height distributions at 14 days, whereas those from thermal neutron irradiation had a narrow range.

Differential killing was a characteristic of the material subjected to the three highest doses of x-radiation. Such a lethality pattern did not occur in the thermal neutron treated material, i.e., if any seedlings survived a treatment, essentially all of them did.

For similar chromosomal aberration frequencies there was less seedling

injury in seeds subjected to thermal neutrons than in seeds treated with x-rays. Apparently x-rays have proportionately more effect on the extra-chromosomal elements of the cell than do thermal neutrons.

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† Published with the approval of the Director as paper No. 571, Journal Series, Nebraska Agricultural Experiment Station.

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THE EFFECTS OF CARBON DIOXIDE ON THE FREQUENCY OF X-RAY INDUCED CHROMOSOME ABERRATIONS IN TRADESCANTIA

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Communicated by Karl Sax, June 24, 1952

The role of oxygen as a factor in radiosensitivity of various cells and tissues has been the subject of considerable study (Sparrow and Rubin,[1] Giles and Riley,[2] Conger and Fairchild,[3] King, Schneiderman and Sax[4]). Little is known concerning the influence of another equally important molecule—carbon dioxide. Although carbon dioxide has for a long time been associated only with photosynthesis and as an end-product of respiratory metabolism, it has become increasingly clear since the classical experiments of Wood and Werkman[5] that carbon dioxide is vitally concerned in the biochemical activity of cells, particularly in synthetic reactions. The experiments to be reported show that carbon dioxide plays a significant role in radiosensitivity and thus reemphasizes the biochemical importance of this gas.

Materials and Methods.—*Tradescantia paludosa* Anderson and Woodson was used in all experiments. Cytological observations were made of chromosomal aberration types 5 days after irradiation. The principal

types of aberrations analyzed at this period are double interchanges (dicentrics and centric rings).

The method used is essentially identical to that described previously (King, Schneiderman and Sax[4]). The experimental material was placed inside a 3460-cc. cylindrical lucite chamber fitted with brass end-plates, gaskets and needle valves. The inflorescences were inserted in an upright position through holes in a waxed cardboard container filled with spring water. The container was placed in the center of the lucite chamber to insure uniform exposure and minimize secondary radiation from the brass end-plates. Dosage was measured with a dosimeter placed at the position of the inflorescences in a lucite chamber identical with the experimental chamber, and placed beside the experimental chamber. In all experiments the dosage rate and total dosage were constant, i.e., 40 r/min. for 10 minutes.

The gases used in the experiments were obtained in commercial cylinders and assayed as follows:

Nitrogen (Airco), 99.5% N_2 plus 0.5% O_2.
Oxygen (Airco), 99.5% O_2 plus 0.5% N_2.
Carbon dioxide (Liquid Carbonic Co.), 99.8% CO_2, 0.05% N_2, 0.009% O_2, 0.07% H_2O.

The inflorescences were enclosed in the air-filled lucite chamber and then compressed to the desired pressure with the gas under consideration. Total gas pressure was measured by a gauge calibrated in ounces per square inch. For pressures below 6% of an atmosphere, measured volumes of specific gases were injected into the air-filled lucite chamber via a rubber policeman fitted on one of the valves. When sealed, the chamber thus contained one atmosphere of air plus a positive pressure of the experimental gas. The average time of compression was 1 minute and the timing of exposure to the gas was calculated from the moment the final pressure was attained. After compression the sealed chamber was kept at 25°C. in the dark for 1 hr. and then placed in the x-ray machine alongside the chamber containing the dosimeter. Irradiation was then performed at room temperature in subdued light. In each experiment this procedure was carried out twice—once with the experimental gas (CO_2) and once with the control gas (N_2 and/or air). The two experimental chambers were compressed and later irradiated within 45 minutes of each other. After exposure the chambers were decompressed slowly for 30 minutes and then opened to air.

In general, from 10 to 15 inflorescences were exposed in each chamber and as many slides as possible made 5 days following irradiation.

Results and Discussion.—The experimental results are presented in table 1. All the scoring was done by one investigator (E. D. K.). The first column in table 1 indicates the pressure of CO_2 superimposed upon the one atmosphere of air initially present in the chamber. The second column

tabulates the number of chromosomes counted. The third indicates the number of dicentrics and centric rings in the experimental preparation. The fourth records the gas composition of the control chamber. In the fifth and sixth columns the number of chromosomes counted and the per cent dicentrics and centric rings found in the control chamber are listed. In the seventh column is tabulated the per cent increase in dicentrics and centric rings of the experimental preparation when compared with the control preparation. In all experiments except the first one listed, the exposure was 40 r/min. for 10 minutes. In the first experiment tabulated, inflorescences were not irradiated. These results may be summarized as follows:

TABLE 1

FREQUENCIES OF DICENTRICS AND CENTRIC RINGS INDUCED IN TRADESCANTIA MICROSPORES EXPOSED TO X-RAYS IN MIXTURES OF AIR, NITROGEN AND CARBON DIOXIDE ALL DOSAGES ADMINISTERED AT A CONSTANT INTENSITY OF 40 R/MIN. FOR 10 MINUTES

(For further discussion, see text)

EXPERIMENTAL				CONTROL				INCREASE OF DICENTRICS AND RINGS OF EXPERIMENTALS OVER CONTROLS, %
GAS MIXTURE (ATMOSPHERES) CO_2	AIR	CHROMOSOMES COUNTED	DICENTRICS AND RINGS, %	GAS MIXTURE (ATMOSPHERES) N_2	AIR	CHROMOSOMES COUNTED	DICENTRICS AND RINGS, %	
1.33	1[a]	3000	0	1.33	1[a]	3000	0	0
0.005	1	3000	8.6	..	1	3000	7.3	17.8
0.02	1	3000	9.9	..	1	3000	7.7	28.6
0.02	1	6000	10.3	0.02	1	3000	7.4	39.2
0.05	1	3000	12.0	0.05	1	3000	7.4	62.2
0.05	1	3000	12.2	..	1	3000	7.7	58.4
0.067	1	3000	12.4	0.067	1	3000	6.9	79.7
0.33	1	3000	13.4	0.33	1	3000	7.3	83.6
0.67	1	3000	18.2	0.67	1	3000	7.1	156.3
1.00	1	3000	23.8	..	1	3000	7.7	209.1
1.33	1	3000	30.5	1.33	1	3000	6.5	369.2

[a] This preparation was not irradiated.

1. Exposure to carbon dioxide without irradiation results in no chromosomal aberrations.

2. Exposure to carbon dioxide during irradiation increases the frequency of chromosome aberrations.

3. This increase in the aberration frequency depends on the carbon dioxide concentration—the greater the carbon dioxide concentration, the greater the increase in aberration frequency. Carbon dioxide concentrations as low as 0.5% of an atmosphere cause a significant increase in the aberration frequency.

These results provide no basis for deciding whether carbon dioxide causes an increase in the initial frequency of x-ray induced breaks, or affects the reunion of broken ends such that recombination is favored over restitution, or both.

At this time any biochemical explanation of the effects of carbon dioxide on x-ray induced chromosome aberrations would be premature. However, since in recent years carbon dioxide has been shown to play a significant role in non-photosynthetic assimilative processes, it seems likely that action of carbon dioxide on radiosensitivity is in some way related to carbon dioxide fixation. An explanation in terms of pH changes seems less likely.

The effects of carbon dioxide on chromosomes described above may in some way be related to the action of this gas on meiosis and mitosis in Drosophila. In this insect high concentrations of carbon dioxide have been shown to increase the frequency of non-disjunction and cause abnormal mitoses by interfering with the migration of chromosomes to the centrosomes.[6]

Whatsoever may be the biochemical explanation of the observations presented, it is evident that carbon dioxide tension is an important factor in conditioning the response of chromosomes to irradiation.

The possible influence of carbon dioxide tension on radiation effects in normal and malignant animal tissues is at present being examined.

* The cytological aspects of this work were conducted by Edward D. King and financed by aid from the Office of Naval Research, Contract No. N8onr-73100. The biochemical aspects of the work were done by Howard A. Schneiderman, an Atomic Energy Pre-doctoral Fellow.

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COMPETITION BETWEEN DROSOPHILA MELANOGASTER AND DROSOPHILA SIMULANS. II. THE IMPROVEMENT OF COMPETITIVE ABILITY THROUGH SELECTION

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Communicated by Franz Schrader, July 1, 1952

Drosophila melanogaster and *Drosophila simulans* are very similar in morphology, genetics and ecology. Several wild strains have been used in population cage competition experiments.[1] At 25°C., in a mixed population of *simulans* and *melanogaster*, the last mentioned is the superior species and it eliminates *simulans* in approximately 100 days. This result has been observed in 19 of 20 cages studied. The exceptional cage proved to be of considerable interest since in it there developed a strain of *simulans* with greatly improved competitive ability.

The exceptional experiment, cage 8 at 25°, was started with 200 females and 100 males of Florida *melanogaster* strain 99 and a total of 133 females and 67 males of Florida *simulans* strains 75 and 76. Fresh food was added in cups on a schedule of 4 cups per week. Each cup remained in the cage for 28 days. Egg samples were taken at intervals and after development

TABLE 1

POPULATION CHANGES IN CAGE 8

DAYS FROM START	MELANOGASTER MALES, %	TOTAL MALES	DAYS FROM START	MELANOGASTER MALES, %	TOTAL MALES
22	52	163	218	54	307
41	55	339	250	69	307
73	98	332	275	85	149
101	84	212	300	90	186
125	75	325	325	95	248
155	81	461	351	98	253
181	60	427	375	100	255

the adult males were counted. In this manner the changes in relative numbers of the two species were followed. The results are given in the upper left graph of figure 1 and in table 1.

During the first 73 days cage 8 was behaving in the expected manner. Subsequently there was an increase in *simulans* and on the 218th day it comprised approximately half of the population. Thereafter *melanogaster* gradually eliminated *simulans*.

The decline in *melanogaster*, which occurred during the 73- to 218-day interval, suggested that either the *melanogaster* had become less able to compete or that a strain of *simulans* with superior competitive ability was being selected. The following experiment was started to test these alternatives.

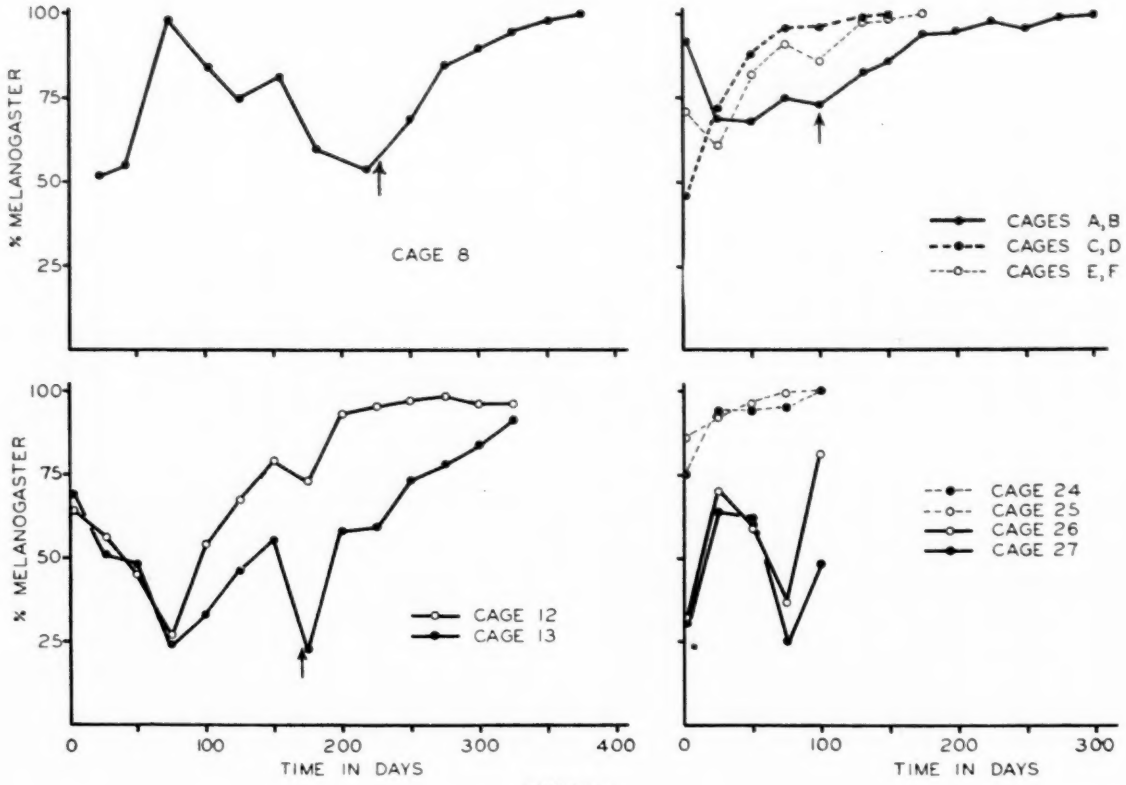

FIGURE 1

Changes in the frequency of *melanogaster* in mixed populations of *melanogaster* and *simulans*. The arrows indicate the time at which the flies were removed for subsequent experiments.

TABLE 2

POPULATION CHANGES IN CAGES A–F. FIGURES ARE PER CENT MELANOGASTER MALES AND TOTAL MALES

DAYS FROM START	A		B		C		D		E		F	
1	89	286	94	198	47	179	45	113	56	111	86	145
25	62	120	76	194	65	212	78	258	51	146	70	117
50	75	247	60	222	80	344	96	327	78	151	85	255
75	84	295	65	380	92	518	99	459	83	352	98	333
100	82	500	63	291	91	489	100	343	74	384	97	408
131	89	253	78	278	98	311	...	...	96	262	99	180
150	84	378	87	283	99	335	...	...	98	334	99.8	357
175	92	286	96	52	99	344	...	...	99.3	272	...	...
200	91	249	98	147	100	270						
225	97	263	100	100								
250	91	211										
275	97	311										
300	100	208										

On the 229th day some adult females were taken from cage 8 and each was put in a separate fly bottle. Since it was not possible to distinguish the females, identification was made on the basis of the F_1 males and then all flies of the same species were put together. This gave a strain of *simulans* that had undergone selection through competition with *melanogaster* for 229 days and a strain of *melanogaster* that had undergone selection through competition with *simulans* for the same length of time. These 229-day selected strains were put into competition with stock strains that had been kept in pure culture. In cages A and B the 229-day selected *simulans* competed with stock *melanogaster;* in C and D the 229-day selected *melanogaster* competed with stock *simulans;* in E and F stock *simulans* competed with stock *melanogaster*. The results are given in table 2 and the upper right graph of figure 1. The *melanogaster* that were selected

TABLE 3

POPULATION CHANGES IN CAGES 12 AND 13

	12		13	
DAYS FROM START	MELANOGASTER MALES, %	TOTAL MALES	MELANOGASTER MALES, %	TOTAL MALES
3	64	245	69	213
27	56	334	51	298
50	45	211	48	160
75	27	195	24	268
100	54	194	33	205
125	67	335	46	273
150	79	236	55	167
175	73	392	23	442
200	93	321	58	244
225	95	358	59	268
250	97	143	73	150
275	98	202	78	239
300	96	157	84	237
325	96	308	91	238

through competition with *simulans* (C and D) were similar to, or identical with, the stock *melanogaster* in competition with stock *simulans* (E and F). The selected *simulans* (A and B) were more successful than stock *simulans* in competition with stock *melanogaster*.

These results suggested that it might be possible to improve the competitive ability of *simulans* through a continuous process of selection. Probably less than 1 per cent of the eggs deposited in cups of a population cage reach the adult stage. With the possibility of such intensive selection, a genotype with improved competitive ability would be at a great advantage.

Some females were taken from cage B on the 100th day and those that proved to be *simulans*, together with their offspring, were combined giving a strain that had been in competition with *melanogaster* for a total of 329

days. This strain was put in competition with unselected *melanogaster* in two cages, 12 and 13. The results are given in table 3 and in the lower left graph of figure 1. It was necessary to terminate these cages after the 325-day sample was collected. It would appear from the results that the 329-day strain of *simulans* was superior to the 229-day strain in competitive ability and it is certainly far better than the unselected *simulans*.

The experiments were continued one step further. Adult females were isolated from cage 13 on the 171st day. From these a strain of *simulans* was obtained that had been selected through competition with *melanogaster* for a total of 500 days. These were put into competition with stock *melanogaster* (cages 26 and 27) and control experiments of stock *melanogaster* with stock *simulans* (cages 24 and 25) were also conducted. It was not possible to continue these cages after the 100-day sample was taken. The results are shown in the lower right graph of figure 1. The selected *simulans* are definitely superior to the unselected *simulans* but it is not clear if the 500-day strain is better than the 329-day strain of the previous experiment.

The results taken as a whole indicate that flies with improved competitive ability can be developed by selection and that this can be done in relatively short periods of time. The importance of this ability for the survival of a species is clear. In a species not living near the limits of its ecological tolerances, competition with other species is one of the main factors influencing its existence. For survival a species must compete successfully. Probably the usual solution is adaptation to a particular niche that becomes its own and thus to limit the ecological area in which it must compete. In the present case it is likely that *simulans* has become better adapted, through selection, to a niche in which *melanogaster* is still superior.

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*A MORPHOLOGICAL BASIS FOR HIGHER NIACIN IN SUGARY MAIZE**

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Previous studies have shown that sugary (su_1) maize endosperms or kernels contain about twice as much niacin as those of the normal starchy (Su_1) allele.[1-3] The data in this paper show that the higher niacin has a morphological basis in that sugary kernels have more niacin-rich aleurone tissue than starchy. This finding makes suggested biochemical explanations[4-5] of the carbohydrate-niacin differences unnecessary.

Methods.—The niacin assays were carried out by use of *Lactobacillus arabinosus* according to the method of the Association of Vitamin Chemists.[6] Assay figures are given on the basis of samples dried at 50°. Carbohydrate was determined as glucose by the Somogyi-Benedict method[7] following acid hydrolysis of samples. Protein values were obtained by multiplying per cent nitrogen by 6.25, the nitrogen being determined by Nesslerization after sulfuric acid-peroxide digestion.

Results and Discussion.—Inasmuch as an ear segregating for sugary and normal shows higher niacin content in both whole and degermed sugary kernels than in the normals, the increase of niacin is not due to the embryos. Nor can it be a difference in the pericarps, since the pericarp is maternal tissue and therefore the same for all kernels of the segregating ear. Therefore differences in niacin distribution were looked for within the starchy and sugary endosperms. Heathcote, Hinton and Shaw[8] who have recently reported data on dissected kernels found approximately 60 per cent of the niacin in the one-cell thick aleurone layer of the endosperm. Unfortunately they did not examine comparable starchy and sugary lines.

In the separation of pericarp, aleurone, inner endosperm, and embryo there was the possibility of niacin diffusion from one tissue to another if water soaking of mature dried kernels was used to prepare seed for dissection. Consequently a different dissection technique was adopted. Two almost mature segregating ears were used which, although sufficiently mature to permit distinguishing the two kernel phenotypes, had not dried too much for peeling off the pericarp and clean removal of the embryo. Studies of niacin in developmental stages of starchy and sugary kernels by Teas, Cameron and Newton[9] indicate that niacin levels at the stage of development of these two selected ears, estimated at 38 days after pollination, would differ little from those at maturity. Both of the ears selected for dissection carried factors for purple aleurone color. Niacin determinations of the kernel fractions are shown in table 1. Pericarps of starchy and sugary are seen to be identical in niacin concentration and almost the same in micrograms of niacin contributed per kernel. Embryos of sugary kernels from both ears had somewhat higher niacin. Sugary inner endosperm from both ears showed almost twice the niacin concentration of starchy, although the contribution of this tissue to the content of the whole kernel is 20 per cent or less. Sixty to 70 per cent of the niacin of the kernel was found in the aleurone. Calculation from these data showed that 89 and 94 per cent of the "extra" niacin by which sugary endosperm exceeds starchy endosperm is localized in the aleurone. Moreover, in the aleurone the increased niacin is not a consequence of higher concentration, but rather of increased amount of aleurone in sugary, there being about twice (1.98 times) as much aleurone in sugary endosperm of ear number 1 and 1.7 times as much in ear number 2 as in the corresponding starchy endosperms.

In interpreting assays of aleurone which represent a separated single cell layer there arises immediately the question of whether sugary and starchy samples are both pure aleurone. The use of colored aleurone stocks should have assured completeness of aleurone removal. However, some contamination of aleurone with the underlying inner endosperm tissue is unavoidable. It seemed possible that because of different endosperm

TABLE 1

NIACIN IN DISSECTED FRACTIONS OF STARCHY AND SUGARY KERNELS FROM TWO SEGREGATING EARS

Each starchy or sugary sample represents a composite from 10 dissected kernels

EAR NO.	KERNEL PART	WEIGHT % DISTRIBUTION		NIACIN MICROGRAMS PER GRAM[a]		NIACIN MICROGRAMS PER KERNEL PART	
		Su	*su*	*Su*	*su*	*Su*	*su*
1	Pericarp	5.4	8.0	7.8	7.8	0.103	0.111
	Aleurone	2.2	6.0	545	372	2.940	3.982
	Inner endosperm	81.6	68.8	3.2	6.3	0.647	0.779
	Embryo	10.8	17.2	18.8	23.7	0.498	0.728
2	Pericarp[b]	..	..	..	..	..	..
	Aleurone	2.1	5.4	289	220	1.590	2.090
	Inner endosperm	86.9	76.3	2.8	4.9	0.627	0.658
	Embryo	11.0	18.3	17.5	18.4	0.505	0.589

[a] Micrograms of niacin calculated for whole kernel samples of *Su* and *su* are 17.1 and 31.4 for ear number 1 and 10.4 and 19.3 for ear number 2.

[b] Not assayed. Pericarp omitted in calculations of ear number 2.

TABLE 2

CARBOHYDRATE AND PROTEIN CONTENT OF ALEURONE AND INNER ENDOSPERM OF STARCHY AND SUGARY KERNEL FRACTIONS

EAR NO.	KERNEL PART	OBSERVED % CARBOHYDRATE		CALCULATED[a] % CARBOHYDRATE, *su*	OBSERVED % PROTEIN		CALCULATED % PROTEIN, *su*
		Su	*su*		*Su*	*su*	
1	Aleurone	19.7	23.3	44.0	26.0	23.8	20.0
	Inner endosperm	71.8	68.8		12.1	13.9	
2	Aleurone	22.8	22.3	40.1	24.0	23.6	20.6
	Inner endosperm	62.6	63.7		12.8	16.1	

[a] Calculated concentration is that per cent expected if the extra amount of aleurone in sugary were a result of contamination of the sample with sugary inner endosperm.

texture the "extra" sugary aleurone might have arisen from contamination with a considerable amount of inner endosperm. If this were true it would be expected that an uncontaminated sample of sugary aleurone would weigh approximately the same per kernel and have about the same composition as a sample of starchy. One should be able to decide by chemical analysis if aleurone of sugary is contaminated with an almost equal weight of inner endosperm, since there is an appreciable difference in the

nitrogen content of aleurone and inner endosperm, and a considerable difference in carbohydrate content. As shown in table 2, the greater amount of aleurone in sugary cannot be explained by differential contamination because such contamination would require that carbohydrate of sugary aleurone be 44.0 and 40.1 per cent, and protein 20.0 and 20.6 per cent. Some differential contamination of sugary aleurone may have occurred in the case of ear number 1, but the amount is inadequate to account for the extra aleurone.

The aleurone layer is completely laid down about 21 days after pollination,[10] at which time starchy and sugary kernels are by visual observation and actual measurement very similar in volume. Since the size of the kernels is approximately the same until they dry with approaching maturity,

TABLE 3

MEASUREMENTS OF ALEURONE CELL THICKNESS IN STARCHY AND SUGARY KERNEL SECTIONS

Thickness in microns of aleurone cells. Each figure given represents the average of ten consecutive cells on each of four sections.

SOURCE OF KERNELS	KERNEL PHENOTYPE	POSITION IN KERNEL		
		CROWN	MIDDLE	SIDE
Segregating ear	*Su*	47.5	35.0	54.5
	su	48.5	45.0	72.0
Segregating ear	*Su*	34.6	32.0	42.4
	su	48.8	46.0	73.9
New occurrence of *su*	*Su*	38.4	31.4	43.7
	su	43.0	39.8	58.0
TB-4a sugary ear	*Su*	36.5	36.3	40.8
	su	52.3	54.9	58.2
Segregating ear	*Su*	28.1	27.0	46.3
	su	50.5	51.6	68.7
Mean	*Su*	46.2	32.4	45.6
	su	60.7	47.4	67.0
Ratio of means	*su*/*Su*	1.31	1.45	1.47

differences in the amount of aleurone per kernel should reflect differences in aleurone thickness. This was tested directly by measuring the thickness of aleurone cells. Starchy and sugary kernels from five segregating ears were fixed, dehydrated, embedded and sectioned according to the procedure developed by Larkin, *et al.*,[11] for maize seed. Kernels were cut before fixation in order to speed up penetration of reagents. Each kernel was divided into halves by a median cut, then divided further by a transverse cut. The two pieces representing the crown of each of three or four kernels were mounted on cardboard by the method of Randolph[12] to assure proper orientation. Sections were cut at 15–25 microns through the central region of the kernels in a plane at right angles to the original median and transverse cuts. Each section therefore represented aleurone cells from

the center of the crown to about half way to the apex of the kernel. Slides were coded and cell measurements taken with an ocular micrometer at a magnification of 440. The thickness of 10 consecutive aleurone cells at the top (center of crown), middle, and side (toward the embryo) portions of each section was recorded. Two sections were examined from each of two slides from each sample of starchy and sugary kernels. Figure 1 is a camera lucida drawing of typical aleurone cells of the two types. The averages for the three classes are shown in table 3. In all cases the sugary aleurone cells averaged thicker than starchy ones. This difference in aleurone thickness was clearly demonstrated by a starchy-sugary connate (twin) kernel sectioned in a longitudinal plane that included the two embryos. In this case the adjacent starchy and sugary aleurone tissue was strikingly different in thickness as seen side by side in the same microscope field. The discrepancy between the measured thickness of sugary aleurone, approximately 1.4 times (table 3), and the amount by weight, about 1.8 times, may be due to differences in thickness of aleurone in the basal half of the kernel which was not included in the sections measured.

FIGURE 1

Camera lucida drawings of typical starchy and sugary aleurone cells.

It is anticipated that other "pleiotropic effects" of the sugary-starchy alleles may owe their origin to differences in aleurone thickness, and also that selection of high niacin maize may involve, at least in part, a selection for thicker aleurone.

Summary.—Approximately 90 per cent of the higher niacin of sugary maize has been found to have a morphological basis in that sugary kernels have a thicker niacin-rich aleurone layer than starchy kernels.

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*THE FOURIER HEAT EQUATION IN RIEMANNIAN SPACE**

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1. Let a certain body, capable of absorbing heat, occupy a region of n dimensional Riemannian space V_n whose metric is defined by the positive quadratic differential form $ds^2 = g_{ij}\,dx^i\,dx^j$. Suppose that this body is heated by conduction in any manner. The situation can be visualized as a flow of heat from the warmer to the cooler parts of the body.

The rate of flow of heat within the body can be depicted as a vector field $X^i(x;\,t)$ or $X_i(x;\,t)$, where $X^i = g^{ij}X_j$ and $X_i = g_{ij}X^j$. At the position x within the body for the particular instant of time t, the vector X is in the direction of the flow of heat and its magnitude is the rate of flow of heat. It is assumed that this vector field $X^i(x;\,t)$ or $X_i(x;\,t)$ is single valued and continuous with continuous partial derivatives of first order over the given region and in a given interval of time.

The lines of flow of heat are the intergral solutions of the system of n ordinary differential equations of the first order

$$\frac{dx^i}{dt} = X^i(x;\,t). \tag{1}$$

In general, there are ∞^n lines of flow of heat.

2. There are two fundamental assumptions which characterize a given flow of heat.[1] These may be described in the following manner.

Assumption 1.—The velocity of the flow of heat at any position x in the body for any given instant of time t, is proportional to the rate of decrease of the temperature U at the position x for the given instant of time t. That is

$$X_i = -k\frac{\partial U}{\partial x^i}. \tag{2}$$

The factor of proportionality k is the conductivity of the body. In certain bodies such as crystals, this k depends not only on the position of the point x and the time t, but also on the direction through the point x. We

shall study only thermally isotopic bodies, where k depends only on the position of the point x and the time t. Of course, it is always supposed that $k > 0$.

The temperature $U = U(x; t)$ is assumed to be single valued and continuous with continuous partial derivatives of at least the second order over the region and in the given interval of time.

Assumption II.—For every n dimensional region R_n within the body, the rate of flow of heat into R_n is equal to the rate of increase of the temperature of R_n.

Let γ_i be the unit normal vector of the closed $(n-1)$ dimensional hypersurface R_{n-1}, which bounds an n dimensional region R_n within the body. The vector γ^i points outwards from R_n. The normal component of the vector field $X^i(x; t)$ along R_{n-1} is $N = X^i\gamma_i$. The rate of flow of heat into R_n is the negative of the flux of the vector field $X_i(x; t)$ out from the bounding $(n-1)$ dimensional hypersurface R_{n-1}, namely

$$-\int_{R_{n-1}} N\, dv_{n-1} = -\int_{R_{n-1}} X^i\gamma_i\, dv_{n-1} = -\int_{R_n} \operatorname{div}(X)\, dv_n. \quad (3)$$

This follows from the Divergence Theorem of Green and Gauss.

If c is the specific heat of the substance, the number of heat units acquired by the body for each unit of mass is $c\dfrac{\partial U}{\partial t}$. If $\rho(x) > 0$, is the density function of the body, then the total number of heat units acquired by the portion of the body in the region R_n per unit interval of time is

$$\int_{R_n} c\rho \frac{\partial U}{\partial t}\, dv_n. \quad (4)$$

Applying Assumption II to (3) and (4), it is found that

$$\int_{R_n} \left[c\rho \frac{\partial U}{\partial t} + \operatorname{div}(X) \right] dv_n = 0. \quad (5)$$

2.1. The temperature U of the body obeys the Fourier heat equation

$$\operatorname{div}\left(k \frac{\partial U}{\partial x}\right) = c\rho \frac{\partial U}{\partial t}. \quad (6)$$

If k, c, ρ are all constant so that the diffusivity $a^2 = k/c\rho$, is constant, the Fourier heat equation is

$$\operatorname{div}\left(\frac{\partial U}{\partial x}\right) = \frac{1}{a^2} \frac{\partial U}{\partial t}. \quad (7)$$

This result is a consequence of equations (2) and (5).

If after a certain lapse of time, the flow of heat becomes stationary so that the temperature U is independent of the time t, then the Fourier heat

equation (7) reduces to the Laplace equation. In this stationary case, it follows from (1) that the flow of heat throughout the body is stationary, and there are ∞^{n-1} lines of flow.

3. By appropriately changing the unit of time, the Fourier heat equation (7) may be written in the form

$$g^{-1/2}\frac{\partial}{\partial x^i}\left(g^{1/2}g^{ij}\frac{\partial U}{\partial x^j}\right) = \frac{\partial U}{\partial t}. \tag{8}$$

If $U(x; t)$ is a non-constant solution of this equation (8), the *heat hypersurfaces*, each of $(n-1)$ dimensions, are defined by $U = U(x; t)$, where U and t are two arbitrary constants. Thus along any one of the heat hypersurfaces, both the temperature U and the time t are constant.[2] In general, there are ∞^2 heat hypersurfaces.

For any given fixed instant of time t, the hypersurfaces defined by the equation $U = U(x; t)$, where U is constant, are said to form an *isothermal family*. In general, for any given instant of time, there are ∞^1 isothermal hypersurfaces, each of $(n-1)$ dimensions. The system of ∞^2 heat hypersurfaces consists of ∞^1 isothermal families of ∞^1 hypersurfaces.

3.1. For any given instant of time, the flow of heat is orthogonal to the family of ∞^1 isothermal hypersurfaces.

This is a consequence of equations (1) and (2).

3.2. Let $F(x; t, U)$ be a single valued and continuous function with continuous partial derivatives of at least the first and second orders over a region of the x's, t, U, such that $\partial F/\partial U$ is nowhere zero in the region. The equation

$$F(x; t, U) = 0, \tag{9}$$

represents the implicit form of the relation between the coordinates x^i of the point, the time t, and the temperature U if and only if F obeys the partial differential equation of second order

$$\left(\frac{\partial F}{\partial U}\right)^2\left[g^{-1/2}\frac{\partial}{\partial x^i}\left(g^{1/2}g^{ij}\frac{\partial F}{\partial x^j}\right) - \frac{\partial F}{\partial t}\right] - 2\frac{\partial F}{\partial U}g^{ij}\frac{\partial^2 F}{\partial U\partial x^i}\frac{\partial F}{\partial x^j} + \frac{\partial^2 F}{\partial U^2}g^{ij}\frac{\partial F}{\partial x^i}\frac{\partial F}{\partial x^j} = 0, \tag{10}$$

where partial differentiation with respect to an x^i or t signifies that U is held fixed.

4. If the number of heat hypersurfaces in a certain flow of heat is merely ∞^1, then the set of hypersurfaces is called a *degenerate heat family*.[3] For a degenerate heat family, the equation (9) can be written in the form

$$f(x) = C(t, U), \tag{11}$$

where f is single valued and continuous with continuous partial derivatives

of at least the first and second orders over a region of the x's and C is single valued and continuous with continuous partial derivatives of at least the first and second orders such that $\partial C/\partial U$ never vanishes over a region of values (t, U).

4.1. A one-parameter system of hypersurfaces, each of $(n - 1)$ dimensions, in a Riemannian space V_n of $n \geqq 2$ dimensions, is a degenerate heat family of hypersurfaces if and only if its equation can be written in the form $f(x) = const.$, such that f obeys a partial differential equation of second order of the form

$$g^{-1/2} \frac{\partial}{\partial x^i} \left(g^{1/2} g^{ij} \frac{\partial f}{\partial x^j} \right) = af + b, \tag{12}$$

where a and b are constants.

This can be deduced after a long discussion from equations (9), (10) and (11).

4.2. Under homothetic representations of Riemannian manifolds, any degenerate heat family can be given by an equation of the form $f(x) = const.$, where f is a solution of one of the three types of partial differential equations of second order:

Type I. The Laplace equation

$$g^{-1/2} \frac{\partial}{\partial x^i} \left(g^{1/2} g^{ij} \frac{\partial f}{\partial x^j} \right) = 0. \tag{13}$$

Type II. The special Poisson equation

$$g^{-1/2} \frac{\partial}{\partial x^i} \left(g^{1/2} g^{ij} \frac{\partial f}{\partial x^j} \right) = 1. \tag{14}$$

Type III. The Helmholtz-Pockels equation

$$g^{-1/2} \frac{\partial}{\partial x^i} \left(g^{1/2} g^{ij} \frac{\partial f}{\partial x^j} \right) = \pm f. \tag{15}$$

This result follows from equation (12).

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THE ISOLATION AND BIOCHEMICAL CHARACTERIZATION OF THE MITOTIC APPARATUS OF DIVIDING CELLS.*

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Communicated by Curt Stern, June 19, 1952

In this communication, the term "mitotic apparatus" (abbreviated MA) will refer to the ensemble of structures constituting the "chromatic" and "achromatic" figures in the classical descriptions of mitosis (e.g., Flemming,[1] Wilson[2]). It includes spindles, asters, centrioles, nuclei (before breakdown) and chromosomal structures (after breakdown of the nuclear membrane). The classical pattern of mitosis describes the coordinated behavior of these elements without implying that they are integrated into a physical entity to which such a term as "mitotic apparatus" could be applied. Our observations, as will be seen, do indicate the existence of just such a physical association of the various elements, permitting their isolation as a single body, and thus the use of the term seems to be justifiable.

Heretofore, the physical and chemical study of the MA has been rendered difficult by the necessity of studying it *in situ*. Most of the earlier information has been adequately summarized by Hughes.[3] The most informative avenues of information have been: (*a*) Staining (e.g., Tahmisian and Brues,[4] Mazia, Brewer and Alfert[5]), (*b*) polarized light studies (e.g., Inoue and Dan,[6] Swann[19]), (*c*) electron microscopy (e.g., Rozsa and Wykcoff[7]), (*d*) hydrostatic pressure studies (e.g., Pease[8]), (*e*) measurements of anaphase movement of chromosomes (Hughes and Swann[9]), (*f*) correlation of dimensional changes of MA with the progress of cytoplasmic division (Dan[10]). An enormous literature on the destruction of the MA by antimitotic agents has yielded little insight into its nature. It is evident that the avenues of information have been entirely visual, and that the MA presents exceptional difficulties because it is a structure without a boundary, whose parts are mingled with other cytoplasmic constituents.

In the following we describe methods for isolation of the MA in quantity from populations of dividing sea urchin eggs. The somewhat unorthodox methods described were developed after numerous failures with procedures involving disruption of living eggs. Such procedures sometimes yield the MA from a few individuals.

Danielli[11] reports briefly on experiences in liberating the MA from eggs by osmotic rupture.

A. The Quantity Isolation of the MA

The instability of the MA is evident in its natural behavior. It is a

structure that seems dramatically to differentiate out of seemingly undifferentiated cytoplasm when division is about to take place and to disappear back into the cytoplasm after division is over. Minor disturbances of the dividing cell cause it to disappear reversibly. It therefore appeared that the best approach to isolation was first to stabilize it, preferably by methods subject to biochemical interpretation.

1. Preparation of Material.—The eggs of the sea urchins *Strongylocentrotus franciscanus* and *S. purpuratus* were employed. In order to obtain pure MA preparations, it was necessary to remove the fertilization membranes and this was accomplished, before fertilization, by treatment for 10 minutes with a mixture containing 1–2 mg. crystalline trypsin and 1–2 mg. crystalline chymotrypsin per 100 ml. of sea water. The eggs were inseminated, permitted to develop to the desired stage of the first division in rather dilute populations, then concentrated by centrifugation.

2. "Fixation."—After trial of numerous procedures it was found that the MA could be preserved by immersion of the eggs in 30 per cent ethanol at about $-10°$C. From the standpoint of protein chemistry, this is not a drastic procedure. Many proteins will be preserved in the native state.

The eggs could be maintained in 30 per cent alcohol at $-10°$ for a period of some weeks, but became more and more resistant to the subsequent treatments on prolonged storage.

The subsequent steps in the isolation were not successful if living eggs were used or if much lower or higher concentrations of alcohol were employed. In the first two cases, the MA apparently disintegrated; when stronger alcohol was used, the cytoplasm coagulated. Therefore, the cold alcohol treatment represents a compromise situation where the MA is stabilized without coagulation of the cell contents.‡

3. Separation of MA.—(*a*) *Mechanical Method:* Eggs preserved by the above method were suspended in water, placed in a large Luer syringe and rapidly forced through a No. 25 syringe needle. The cytoplasm was largely dispersed, and the MA separated from it. If whole eggs remained, the process was repeated. The MA could then be concentrated by repeated slow centrifugation (Servall SS-1 centrifuge operated at 30 volts), or by sedimentation under gravity, discarding the cytoplasmic granules which remained in the supernatant fluid. Figure 1, Plate 1, shows the appearance of the MA isolated by this method, seen by phase contrast. In this photograph, an isolated MA lies beside an unbroken egg, and the appearance of the MA before and after isolation is evident.

The MA as isolated by this method is rather shrunken, sticky and appears to be contaminated with adhering cytoplasmic material. On close examination, however, the fibrous structure of the spindle region is clearly evident (Fig. 2). If it is an artefact, the artefact results from treatment that is very much milder (from the standpoint of protein chemistry) than the methods of cytological fixation.

The MA prepared by this method may be freed from cytoplasmic particles by washing in alkaline water (pH *ca.* 9).

(*b*) *Solubilization Methods:* Considering the possible structure and mechanisms of formation of the MA, an obvious possibility is that it is composed of fibrous proteins which, whether formed by unfolding or by polymerization of corpuscular units, might differ in solubility from other proteins present. In tests with various solubilizing procedures, it was evident that detergents provided a means of dispersing the cell contents, but, with eggs taken from 30 per cent alcohol, the MA also dissolved. It seemed possible, from precedents that need not be discussed here, that the establishment of an increased number of S–S bonds might increase the stability of the fibrous MA toward solubilization by detergents, and this proved to be the case.

Briefly, it was found that if the cells, previously preserved in cold 30 per cent alcohol, were treated with agents capable of oxidizing –SH groups, the surrounding cytoplasm could simply be dissolved (or, more properly, dispersed) by detergent, leaving the intact MA behind. The agents tested were H_2O_2, I and ferricyanide. All worked, in the sense of making the MA resistant to detergent, but H_2O_2 proved to be best for our purposes because it did not coagulate the cytoplasm. Iodine and ferricyanide rendered the cytoplasm resistant to detergent in the same way as did higher concentration of alcohol or warming; it swelled and became more transparent, but did not disperse into a homogeneous suspension.

A number of detergents was tested, including Nacconol NRSF, Tween, Duponol C, Duponol D, Duponol G, Dupo nol LS and Duponol ME. Duponol C and Duponol D were finally selected. The former liberated the MA effectively, but in a highly hydrated form that was difficult to observe even in phase contrast. It could be partially dehydrated by addition of electrolyte, but it proved to be more practicable to use Duponol D, a sodium lauryl sulfate preparation containing some electrolyte.

Before describing the details of the method, certain points should be clarified. (1) The cytoplasm is dispersed but not entirely dissolved; a suspension of fine particles is recovered. (2) The isolation of the MA by the selective action of detergent is possible only after previous "fixation" in subzero 30 per cent ethanol and treatment with an oxidizing agent. (3) The fact that the treatment with an oxidizing agent produced the desired result does not in itself imply that the reasoning behind the procedure is correct.

While the "fixation" procedure described was essential, its very mildness created a complication. Catalase apparently was not denatured, and when the "fixed" eggs were treated with peroxide a great deal of foaming resulted. Thus the final concentration of peroxide cannot be defined without analysis. Where eggs had been in storage too long, and catalase activity was much

reduced, the cytoplasm was correspondingly more resistant to dispersal, and the isolation less successful.

Details of two procedures will be given, which we have termed the "low-peroxide" and "high-peroxide" methods. The difference between the methods tends to support the reasoning underlying their development. After exposure to a low concentration of peroxide, the MA may be isolated by using a small volume of Duponol for a short time, but slowly dissolves in larger amounts of Duponol. After treatment with a higher concentration of peroxide, the MA is isolated only by use of a large amount of detergent for a longer time. The behavior is determined by the amount rather than concentration of detergent, confirming the statement of Putnam[12] that the solubilization of proteins by detergents is a function of weight-to-weight relationships rather than of concentration as such.

(*1*) *"High-Peroxide" Method:* The fixed eggs are removed from the cold chamber and immediately mixed with an equal volume of 12–15 per cent H_2O_2. There is considerable foaming, but the evolution of oxygen declines as the catalase becomes inactive. The eggs are left in this mixture for 30 minutes at room temperature. In phase contrast, the fibrous elements of the MA appear sharper and more refractile than before treating with H_2O_2. To the mixture, 20–25 volumes of 1–2 per cent Duponol D in water are added. The mixture is left at room temperature for 3 hrs. The liberation of the MA is aided by stirring, but in any case the cytoplasmic granules spontaneously disperse, leaving the free MA. At the end of the period the MA, which are much heavier, are collected by centrifugation at 500 g. for 3 minutes (Servall SS-1 at 30 volts). In cases where undissolved eggs are present (e.g., where process of removal of fertilization membrane was not completely effective) these still heavier bodies may be removed by centrifugation at 500 g. for 1 minute. (Times given are periods during which the centrifuge motor was energized, not times at maximum speed.)

The MA preparation is purified by resuspending in water, and centrifuging again in the same way. It is advisable to repeat this at least 3 or 4 times, not only to remove the last granules but also to remove the excess detergent, which forms tactoids at low temperatures and inconveniences observation and analysis. The suspension of MA in water may be stored for several weeks at refrigerator temperatures without drastic changes in morphology.

(*2*) *"Low-Peroxide" Method:* In this procedure the eggs are placed in an equal volume of 3–4 per cent H_2O_2 for 30 minutes at room temperature. To this volume, an equal volume of Duponol D (concentrations ranging from 1 to 2 per cent have proved effective) is added. The dispersal of cytoplasm is slower than in a larger volume of Duponol, and vigorous shaking may be employed advantageously without danger of damaging the MA. Following 30 minutes treatment with Duponol, the MA are separated and washed free of cytoplasmic particles and detergent by the procedure described above.

4. Purity and Yield.—Figure 3, Plate 1, shows the appearance of a typical concentrated suspension of MA obtained by the peroxide-detergent methods. Generally there is a slight contamination by cytoplasmic particles, which may be minimized by repeated washing. Close attention has not been paid to yield, but in typical experiments about 10 mg. of MA protein was obtained from 10 ml. of concentrated eggs most of which were in metaphase

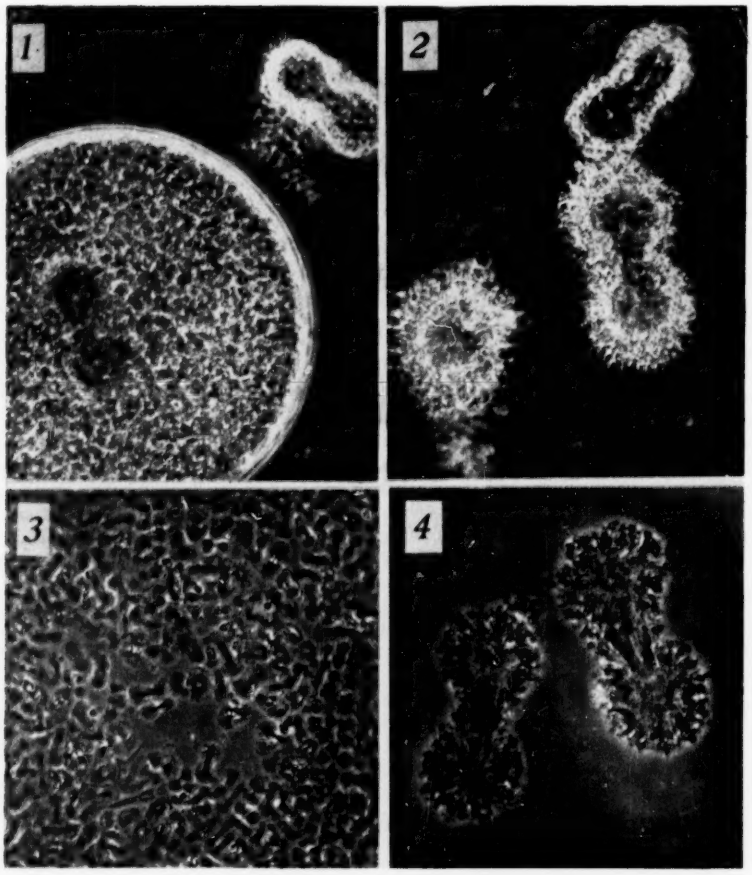


PLATE 1

Fig. 1: Mitotic apparatus of *Strongylocentrotus franciscanus* at metaphase. Material "fixed" in sub-zero 30 per cent ethanol and dispersed mechanically in water. One MA remains inside unbroken egg, the other is isolated from an egg whose cytoplasm has been dispersed. × 240.

Fig. 2: Mitotic apparatus isolated by mechanical method at metaphase and later stages. Fibrous structures more clearly shown than in figure 1. × 240.

Fig. 3: Lower power photograph of a mass preparation of MA. Resting nuclei are derived from unfertilized eggs or fertilized eggs whose divisions are retarded. × 60.

Fig. 4: MA isolated by "high peroxide"-Duponol method. Spindles and cross sections of asters with centrioles are shown. Chromosomes present but not brought out by photograph. × 280.

Figures are photographs of unstained material in water, taken with aid of phase contrast microscope.

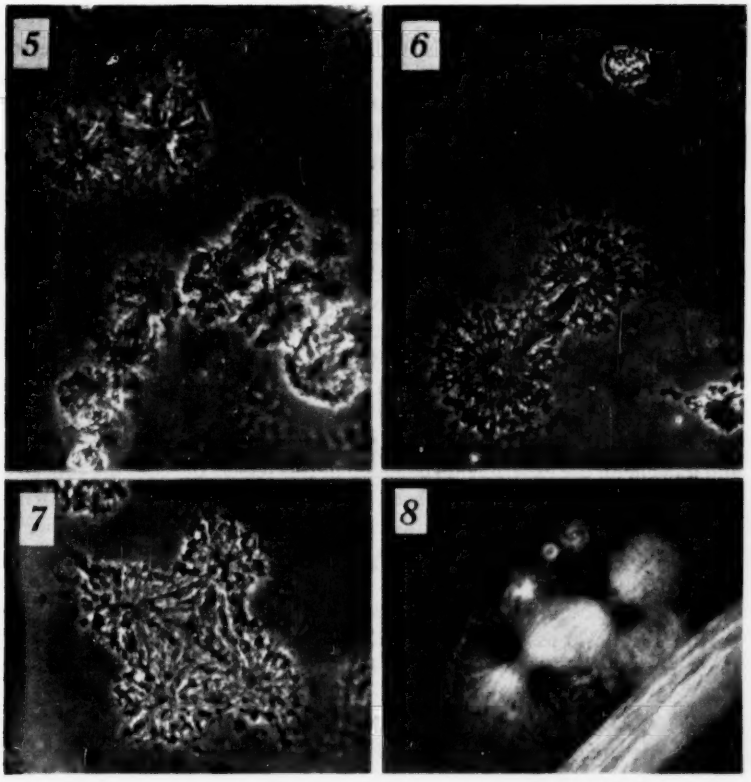


PLATE 2

Fig. 5: Preparation similar to figure 4, but under slight compression to show fibrous structure of asters. × 280.

Fig. 6: Prophase MA (upper) shows two small astral systems attached to prophase nucleus with intact nuclear membrane. Telophase MA (lower) has a longer spindle and larger asters. Nuclei are reconstituting near centers of aster. Demarcation line at equator of spindle is not an equatorial chromosome plate but the "midbody" ("high peroxide"-Duponol preparation). × 280.

Fig. 7: Tetraster spindle derived from polyspermic egg of *S. franciscanus*. × 280.

Fig. 8: Birefringence picture of isolated MA, showing positive birefringence along fiber axis, as in the living. (Courtesy of Dr. S. Inoue.)

Figures 5–7 are phase contrast photomicrographs.

B. The Structure of the Isolated MA

1. Cytological Features.—The isolated MA is rather difficult to detect by ordinary microscopic illumination, and all observations and photographs have employed phase contrast microscopy.

The following statements will refer primarily to MA prepared by the peroxide-detergent methods. In general, the MA as isolated conforms in structure to the picture described in the classical cytological observations on sea urchin eggs (e.g., Wilson[13]). Figure 4, Plate 1, shows several isolated MA from cells in metaphase. The asters, spindles and spindle fibers, and centrioles are evident. The chromosomes also are retained, at least through the earlier stages of isolation, but are rather small and do not appear distinctly in the photographs. The astral rays do not show quite so sharply in figure 4 because of interference by those oriented along the microscope axis, and are much clearer in figure 5, which is a similar preparation but slightly compressed.

The synchrony of the division of a population of sea urchin is not perfect, and if, as in all the experiments included in this paper, the cells are collected when the majority are in metaphase, some will be in stages that are earlier or later than this. Cases of earlier stages are clearly evident in figure 3, Plate 1, since the presence of nuclei with membranes is rather conspicuous. Figure 6 shows a field which contains the MA of a cell with the nuclear membrane remaining and one in telophase. In the former, one sees the growing asters in association with the nucleus. In the latter, it is clear that the asters are larger than in the earlier stages shown in figures 3 and 4 and near the center of the asters the reconstituting nuclei are seen. An, interesting feature of this telophase MA is the suggestion of a plate across the spindle equator, which may correspond to the "midbody" of the classical descriptions (Wilson[2, 13]).

The behavior of the MA as a single physical entity applies even to mitotic abnormalities such as multipolar spindles. Figure 7 shows an isolated tetrapolar MA resulting from polyspermy. Such configurations of the MA retain their interest because of the difficulties they create for simplified theories that reject the reality of the fibrous elements and appeal to plus-minus attractions, fields of force or tactoid formation.

From the cytological standpoint, the observations thus far permit a number of conclusions. Perhaps the most significant is that the *whole MA, including chromatic and achromatic, nuclear and cytoplasmic elements behaves as a single unit in the isolation procedure.* It is conceivable, of course, that this physical association is some artefact of the method, but the normal expectation would have been that the method would result in the disruption of such associations as that between asters and spindles or asters and fusion nuclei. In addition to the suggestion that the elements of the MA are associated into a single physical entity, the results define a chemical

order of unity. Since the method depends on differential resistance to solubilization by detergent, it might have been predicted that some elements of the MA would be preserved while others were dispersed. Instead, we obtain the complete apparatus as defined cytologically.

Occasionally asters were separated from the spindles as a result of handling. Here one often observed in the aster an "insertion cone" where the spindle had been attached, while the spindle poles thus freed from the asters showed convergent ends. In no case were "half-spindles" recovered. It can definitely be said that there are spindle elements which are continuous from pole to pole at all the stages of anaphase and early telophase and are not broken by the mechanical stresses to which they are subjected in these procedures.

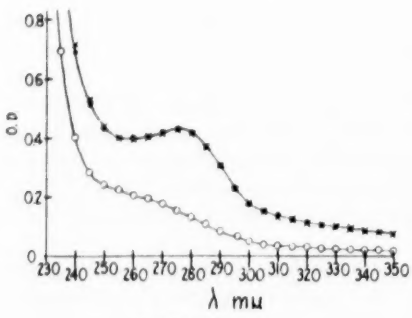
2. The Question of Artefact.—Progress toward an understanding of the mitotic apparatus has been deterred by seemingly endless discussion of artefacts of cytological technique (summarized by Hughes[3]). The introduction of the electron microscope has merely reduced the size of the alleged artefacts and reintroduced purely visual criteria of "good" and "bad" fixation that had become obsolete in light microscopy. The controversy about the reality of spindle fibers will probably be settled by observations such as Inoue's[14] polarization-microscopic records of their appearance in living material.

In the case of the sea urchin egg, the subject of the present study, a number of the elements of the MA are observable in the living,[6] though obscured by cytoplasm, and, in the development of the present isolation technique, it was possible to follow the major structures through all stages from the living to the final isolated product. Following such a procedure, it is clear that no structures are created in the process, although the structural details may conceivably be modified. The successful isolation of the whole MA as a stable coherent structure would seem to eliminate such views as that the astral radiations are fluid channels outlined by cytoplasmic granules or that the spindle fibers are lines of precipitation due to mechanical stress. At least, a mechanism whereby treatment with subzero 30 per cent alcohol would transform the postulated liquid channels or lines of stress into strong fibrous units possessing characteristic arrangement and dimensions at each stage of mitosis is not obvious. On the contrary, the findings are in accord with the notions of Dan and collaborators[6,15] concerning the physical nature of the spindle and aster.

Perhaps the question of the "normality" of the structure obtained by isolation is not meaningful; it still contains an element of cyclic reasoning since the norm is practically derived from fixed material. More significant is the very fact of the isolation itself, especially by the peroxide-detergent procedures, for this defines the MA as a coherent ensemble of structures sharing some chemical properties that differentiate them from everything

else in the cell. Taking the structural and the chemical differentia of the MA together, it is concluded that we can no longer question the tangible reality of the major elements in the classical description of the MA, though we may question dimensional details.

Rozsa and Wyckoff's observation of the absence of distinct spindle fibers (in dividing plant cells) after formalin fixation is not applicable to the dividing sea urchin eggs. After fixation in Baker's neutral formalin and treatment with detergent, the spindle structure appeared essentially as in the isolated spindles. We could not isolate the formalin-fixed MA. While the detergent rendered the coagulated cytoplasm swollen and much more transparent, it did not disperse it.



TEXT FIGURE 1

The ultra-violet absorption of the protein of the mitotic apparatus. Upper curves show the absorption spectra at neutral pH of the material recovered from the first (solid circles) and second (crosses) precipitation. Lower curve shows the absorption of the supernatant (including sodium acetate and residual Duponol) from the first precipitation. Water blank.

3. Birefringence. — Thanks to the cooperation of Dr. Shinya Inoue, of the University of Washington, polarized-light observations were made with his microscope. Figure 8 shows the birefringence of the isolated MA. The sign of birefringence is positive with respect to fiber axes, and the appearance is essentially the same as is observed in the living.[6, 11]

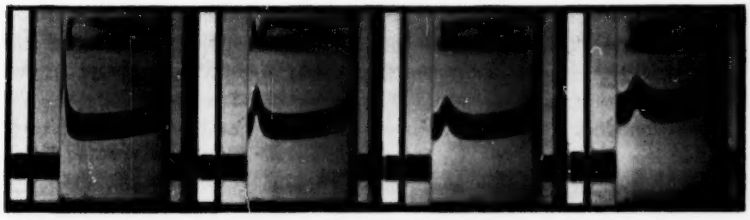
C. Chemical Properties.

1. Separation of MA Protein.—The MA prepared by the peroxide-detergent method is relatively resistant to solution. It is soluble in strong (*ca.* 0.5 *N*) NaOH, and the following data concern the material obtained after solution in this reagent. On back-titration with HCl, an amorphous white precipitate appears at about pH 6, which dissolves as the pH is taken still lower. It is presumed that the region of pH 6 represents the isoelectric point of the protein separated, but the exact point of maximum precipitation has not yet been determined. In most of the experiments, acetic acid was employed to precipitate the MA protein. The precipitate may be redissolved at pH 7–8 by suspension in water and addition of alkali, and reprecipitated by addition of acetic acid to pH 6.

Protein Content: Determinations of the protein content of MA suspensions were made by the quantitative biuret method (Robinson and Hogden[16]). From the biological standpoint, it is most interesting to

determine the amount of protein isolated per unit MA. Crystalline bovine serum albumin was used as a standard. The number of MA per unit volume of suspension was determined by the use of a hemocytometer. This was compared with the amount of protein per egg. In the case of the egg of *Strongylocentrotus franciscanus*, the amount of protein recovered after two isoelectric precipitations was 2.4×10^{-6} gm. per MA. The amount of protein per egg was 1.4×10^{-4} mg. Thus, about 2 per cent of the protein present is used in the formation of the MA. These figures have only order-of-magnitude significance, as the accuracy of the counts is not very high.

2. *Ultra-violet Absorption.*—Text figure 1 shows the ultra-violet absorption curves of the MA protein after the first and second isoelectric precipitations, the material being redissolved each time by addition of sufficient alkali to bring to a pH of about 7.5. The two upper curves coincide, show-



TEXT FIGURE 2

The ultra-centrifugal pattern of the protein recovered from isolated MA after 2 precipitations. Concentration approximately 0.5 per cent. Neutral pH. Pictures taken at 569, 1530, 2490, and 3450 seconds after reaching speed. (59,780 r. p. m.)

ing complete recovery of the protein on the second precipitation. The absorption spectra, with minima at 255 mμ and a maximum at 275 mμ are those of typical proteins. There is no evidence of other peaks. The lower curve is that of the supernatant solution after the first precipitation. The curve is comparable with the upper curves, since the readings were made at the same dilutions, against the same water blank. The lower curve shows no peaks, and, while it may represent absorption by constituents of the MA that were not precipitated at pH 6, it also will include absorption at short wave-lengths by acetate and by whatever Duponol remained in the preparation. Obviously the protein isolated by precipitation at pH 6 accounts for the major part of the UV-absorbing material.

The absence of a nucleic acid peak is not necessarily surprising. At least, the DNA that might be present in the chromosomes (2.3×10^{-9} mg. per diploid set) would not be in sufficiently high concentration to be detectable under the conditions of these measurements.

3. Ultra-centrifugation.—The sedimentation pattern of a 0.5 per cent solution of twice precipitated MA protein was obtained with the aid of a Spinco analytical ultra-centrifuge. We thank Mr. Richard Thomas for making these determinations. The results are shown in text figure 2. Only one boundary is apparent. The sedimentation constant was approximately 4.0 svedberg units. A possible particle weight was estimated, making the assumption of spherical shape and 30 per cent hydration. The weight comes out to be of the order of magnitude of 45,000.

The presence of a single boundary is impressive, in view of the morphological complexity of the MA, but need only imply that other colloidal particles are present only in small amounts. The most reasonable first conclusion would be that we are observing the behavior of a protein constituting the asters and spindle, which accounts for the bulk of the MA. It would be implied that asters and spindles are composed of the same protein even though there is much cytological evidence that they differ in behavior.

The apparent small size of the particles obtained by dissolution of the MA is surprising at first sight, but finds an analogy in the case of actin of muscle, which forms fibrils by polymerization of relatively small particles.

In further work, the MA protein will have to be obtained in solution by a method less drastic than treatment with strong alkali before conclusions allowing physiological interpretations can safely be drawn.

4. The Role of S–S Linkages.—The MA isolated directly from alcohol is soluble in Duponol. After treatment with dilute H_2O_2 it is less soluble, and after concentrated H_2O_2 quite insoluble. This relationship suggested that the stability toward Duponol is related to the number of S–S linkages present, but the practical success of the reasoning does not necessarily prove that it is correct.

If the interpretation is correct, then reduction of S–S linkages should render the isolated MA more soluble in detergent. To test this, MA isolated by the peroxide-Duponol procedures were treated with a 5 per cent solution of sodium thioglycollate, at pH 8. The MA did not dissolve, although they gave a visual impression of softening, curling, etc. If 1 per cent Duponol was then added, the MA dissolved completely. Alternatively, a solution containing 5 per cent thioglycollate and 1 per cent Duponol may be used. The MA dissolves immediately in this. Therefore, the characteristic action of H_2O_2 on the stability toward detergent may be reversed by a treatment that would reduce S–S groups.

The alkaline thioglycollate solution used by Goddard and Michaelis[20] to dissolve wool did not dissolve the MA, but made it visibly more flexible and more soluble in stronger alkali and in Duponol. This might suggest that the essential morphology of the MA does not depend on S–S linkages alone.

These results are not surprising in view of the role of S–S linkages in other types of fibrous structures, but do strengthen the view held by a number of workers since Rapkine (summarized by Brachet[17]) that the oxidation and reduction of S plays an important role in mitosis.

D. Summary

1. Methods have been developed for isolating the mitotic apparatus from dividing sea urchin eggs (*Strongylocentrotus franciscanus* and *S. purpuratus*) in quantity.

2. The structure of the MA isolated at various stages of the mitotic cycle corresponds in essential respects to the classical cytological descriptions.

3. The entire MA, including the "chromatic" and "achromatic" elements, behaves as a single physical entity at all stages observed.

4. The isolation processes based on selective solubilization of the surrounding cytoplasm differentiate the MA from the former on chemical as well as morphological grounds.

5. The isolated MA is positively birefringent with respect to fiber axes.

6. A protein has been separated from the MA by isoelectric precipitation. It accounts for most of the mass of the MA and for about 2 per cent of all the protein of the egg.

7. The MA protein gives a single boundary in the ultracentrifuge, and the sedimentation rate suggests a small particle (particle weight *ca.* 45,000).

8. Experiments demonstrating the role of S–S linkages in the stability of the MA are described.

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† Department of Biology, Tokyo Metropolitan University, Tokyo, Japan. This work was done during tenure of a U. S. Public Health Service Special Fellowship.

‡ It is our present opinion that the subzero ethanol solution is effective not because it alters the MA but because it prevents those reactions which, in the living cell, cause the disappearance of the MA under many abnormal conditions. The procedure may be compared to Szent-Györgyi's[18] technique of preserving the contractile system of muscles by soaking them in cold glycerol solutions.

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